

**International Symposium
The Nucleolus
Its Structure and Function**

nci

MONOGRAPH 23

1977

14-2 3/10/1
LIBRARY

AUG 21 1970

NATIONAL INSTITUTES OF HEALTH

U.S.

NATIONAL CANCER INSTITUTE MONOGRAPH 23

DECEMBER 1966

International Symposium
" ^{on} **The Nucleolus**
Its Structure and Function

Papers + discussion

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NATIONAL CANCER INSTITUTE, BETHESDA, MARYLAND

NATIONAL CANCER INSTITUTE MONOGRAPHS

KENNETH M. ENDICOTT, *Director, National Cancer Institute*

The proceedings of conferences and symposia dealing with cancer or closely related research fields and series of papers on specific subjects of importance to cancer research are presented in these monographs. Send an original, 1 carbon copy, and 2 sets of illustrations (1 mounted) to the Scientific Editor, National Cancer Institute, Bethesda, Maryland 20014. If there is a Conference Editor, manuscripts should be submitted to him.

Text: Double-space all material. Block title and byline on right half of page. Official title or academic degree is optional. Align first word of text with first word of title. In typing, do *not* split a paragraph from one page to another—even if a large amount of blank space is left at the end of the last full paragraph on a page. Type tables on separate pages immediately after their description in text. Insert footnotes as separate paragraphs immediately after they are mentioned. Follow *Webster's International Dictionary* in matters of spelling, capitalization, punctuation, and hyphenation. Follow *Chemical Abstracts* for chemical terms.

References: Double-space and number in order of citation in both text and reference page. Give author, title, journal, volume, first and last page numbers, and year. For book references, give chapter titles when referred to, and name and location of publisher.

Illustrations: Refer to graphs, charts, and line drawings as text-figures and number consecutively throughout text. Type legends in text immediately after paragraph in which text-figures are described. Make lettering on art uniform and large enough to be legible if reduced. Original drawings in black India ink or glossy prints are acceptable. Orient horizontally instead of vertically.

Photographs are printed at the end of each article. Refer to them as figures and number consecutively throughout text. Trim each photograph to show only important area. Maximum size of page for photographs is $5\frac{1}{4} \times 6$ inches. Mount and number one set on white cardboard, with as many figures to a page as possible. Reproduction is best when prints are from negatives of evenly illuminated subject in which details are sharp.

BOARD OF EDITORS

HOWARD B. ANDERVONT, *Scientific Editor*

JEROME B. BLOCK,

Associate Editor

JOSEPH F. FRAUMENI, JR.,

Associate Editor

LAURENCE R. DRAPER,

Associate Editor

MEARL F. STANTON,

Associate Editor

JACQUELINE J. K. WHANG, *Associate Editor*

EDITORIAL STAFF

DORIS M. CHANEY, *Managing Editor*

VIVIAN J. HESTON,

Assistant Managing Editor

MARIE K. MURPHY,

Monograph Editor

Assistant Editors: MERCEDES B. LA CHARITY, ALICE McA. LEE, and
JOAN O'BRIEN

INTERNATIONAL SYMPOSIUM
ON
THE NUCLEOLUS,
ITS STRUCTURE AND FUNCTION, *Montevideo, 1965*
[Papers and discussion]

Held at
Montevideo, Uruguay
December 5-10, 1965

Edited by
W. S. VINCENT
and O. L. MILLER, JR.
Associate editors
M. E. DRETS
and F. A. SAEZ

Acknowledgments

Travel of participants from the United States was partially supported by the U.S. National Science Foundation and the U.S. Atomic Energy Commission. Partial support of travel of Latin American participants was given by the Organization of American States. The Uruguayan authorities contributed partial support for lodging Latin American participants in Montevideo.

The U.S. Atomic Energy Commission also provided partial support for the costs of editing these Proceedings and support for the purchase and distribution throughout Latin America of more than one thousand copies of this volume.

The University of Pittsburgh generously was responsible for dispersing the Symposium funds.

The editors also wish to thank Dr. Mona Coetzee and Mrs. Mary Frances Bradley of Pittsburgh and Mrs. Barbara Beatty of Oak Ridge for their editorial and secretarial assistance.

International Symposium on the Nucleolus—Its Structure and Function

Sponsored by

Consejo Departamental de Montevideo, Uruguay
Consejo Nacional de Investigaciones Científicas y Técnicas, Uruguay
Universidad de la República, Uruguay
Facultad de Medicina de Montevideo, Uruguay
Facultad de Humanidades y Ciencias de Montevideo, Uruguay
Facultad de Veterinaria de Montevideo, Uruguay
Comisión Nacional de Turismo, Uruguay

Biology Division, Oak Ridge National Laboratory
Institute for Cancer Research, Philadelphia
Pan American Union (Organization of American States)
U.S. Atomic Energy Commission
U.S. National Science Foundation
University of Pittsburgh

Organizing Committee

Honorary President: Clemente Estable, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

President: Alexander Hollaender, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Vice-President: Francisco A. Saez, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

Members: Máximo E. Drets, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

Mary Esther Gaulden, Southwestern Medical School, University of Texas, Dallas

Gabriel Gerard, Facultad de Humanidades y Ciencias de Montevideo, Uruguay

O. L. Miller, Jr., Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Crodowaldo Pavan, Departamento de Biología Geral, Universidade de São Paulo, São Paulo, Brasil, and the Biology Division, Oak Ridge National Laboratory, Oak Ridge Tennessee

R. P. Perry, Institute for Cancer Research, Philadelphia, Pennsylvania

Heraldo Poletti, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

J. Roberto Sotelo, Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay

J. I. Valencia, Laboratorio de Genética, Universidad de Buenos Aires, Buenos Aires, Argentina

W. S. Vincent, Department of Anatomy and Cell Biology, University of Pittsburgh, Pittsburgh, Pennsylvania

R. C. von Borstel, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

TABLE OF CONTENTS

Introductions	Page
Alexander Hollaender	xi
Clemente Estable	xv

Symposium Orientation

Chairmen: Clemente Estable and J. R. Totter

Perspectives for the Conference on the Nucleolus	
Jack Schultz	1

Nucleolar Structure

*Chairmen: Gustavo Hoecker, F. J. S. Lara,
Jack Schultz, and J. H. Taylor*

Ultrastructural Aspects of the Normal and Pathological Nucleolus in Mam- malian Cells	
W. Bernhard	13
Changes in Nucleolar Ultrastructure Associated With Differentiation in the Root Apex	
Beal B. Hyde	39
Structure and Composition of Peripheral Nucleoli of Salamander Oocytes	
O. L. Miller, Jr.	53
Ultrastructure and Mode of Formation of the Nucleolus in Plant Cells	
J. G. Lafontaine and Fr. A. Lord	67
Association of Nucleolus and Sex Chromosome in <i>Gryllidae</i> Spermatocytes	
J. Roberto Sotelo and Rodolfo Wettstein	77
Morphology, Structure, and Dynamics of the Nucleolonema	
Clemente Estable	91
Morphological Observations on the Nucleolus of Cells in Tissue Culture, With Special Regard to Its Composition	
Renate Lettré, Wilhelmine Siebs, and Neidhard Paweletz	107
Nucleolar Architecture in Root Meristematic Cells of <i>Allium cepa</i>	
L. A. Chouinard	125
Nucleolar-Chromosomal Interaction in Microspores of Maize	
Hewson Swift and Barbara Jean Stevens	145

Anisonucleolinos in Mammalian Cell Cultures Robert Love	Page 167
Detection and Origin of Nucleolar Components: A Model for Nucleolar RNA Function C. J. Tandler	181

Nucleolar Composition

Chairmen: Giorgio Schreiber and P. P. Cohen

Composition of Nucleoli Isolated From Mammalian Cells Harris Busch, Raoul Desjardins, Donald Grogan, Ken Higashi, Samson T. Jacob, Masami Muramatsu, Tae Suk Ro, and William J. Steele ...	193
Progress Report on the Characterization of Nucleoli From Guinea Pig Liver Rachele Maggio	213
Composition of the Nucleolus as a Basis for Views on Its Function Jan-Erik Edström	223
Proteins and Nucleic Acids of Starfish Oocyte Nucleoli and Ribosomes W. S. Vincent, Elyane Baltus, Arne Løvlie, and R. E. Mundell	235
Synthesis of Transfer RNA in the Nucleolus of <i>Smittia</i> J. L. Sirlin, J. Jacob, and M. L. Birnstiel	255
Autoradiographic Studies of Transfer RNA Metabolism in <i>Vicia faba</i> Root-Tip Cells Philip S. Woods	271
Nucleolar Enzymes of Isolated Rat Liver Nucleoli Günther Siebert	285

The Nucleolus in the Cell Cycle and During Development

*Chairmen: M. E. Drets, J. I. Valencia,
R. C. von Borstel, and Gabriel Favelukes*

The Nucleolus and Synthesis of Ribosomal RNA During Oogenesis and Embryogenesis of <i>Xenopus laevis</i> Donald D. Brown	297
Metabolism of the Nucleolus During Oogenesis, Maturation, and Early Steps of Embryonic Development in Echinoderms Adrienne Ficq	311
Synthesis of Basic Proteins and Cellular RNA Species During Sea Urchin Development Donald G. Comb and David J. Silver	325
Nucleolar RNA Synthesis During Mitotic and Meiotic Prophase Nirmal K. Das and Max Alferf	337
Amino Acid Incorporation Patterns During the Cell Cycle of Synchronized Human Tumor Cells Frederick H. Kasten and Fredy F. Strasser	353

	Page
Altered Patterns of RNA Metabolism in Liver Cells Following Partial Hepatectomy and Thioacetamide Treatment Ruth G. Kleinfeld.....	369
Autoradiographic, Biochemical, and Ultrastructural Studies into the Effect of Actinomycin, 5-Fluorouracil, and Adenosine on Nucleolar and Cellular Structure and Function Unne Stenram.....	379

Nucleolar Genes

Chairmen: Crodowaldo Pavan and Barbara McClintock

Diversity and Variation of the Nucleolar Organizing Regions in Chironomids C. Pelling and W. Beermann.....	393
Problems in the Developmental Cytogenetics of Nucleoli in <i>Xenopus</i> H. J. Barr.....	411
Autoradiographic Studies of RNA and Protein Synthesis in Anucleolate <i>Xenopus</i> Embryos H. Wallace.....	425
Localization of the Ribosomal DNA Complements in the Nucleolar Organizer Region of <i>Xenopus laevis</i> M. L. Birnstiel, H. Wallace, J. L. Sirlin, and M. Fischberg.....	431
On the Chromosomal Distribution of DNA Complementary to Ribosomal and Soluble RNA F. M. Ritossa, K. C. Atwood, D. L. Lindsley, and S. Spiegelman.....	449

The Nucleolus and Ribosome Biogenesis

Chairmen: Carlos Basilio and H. W. Lewis

Nuclear RNA of the Salamander Oocyte Joseph G. Gall.....	475
RNA Metabolism in the HeLa Cell Nucleus and Nucleolus S. Penman, I. Smith, E. Holtzman, and H. Greenberg.....	489
The Nucleolus and Ribosomal RNA Synthesis in Mammalian Cells G. P. Georgiev.....	513
On Ribosome Biogenesis Robert P. Perry.....	527
Panel on Ribosome Biogenesis <i>Panel: M. Birnstiel, D. Brown, J. Gall, S. Penman, R. Perry (moderator), and W. Vincent.....</i>	547

Summation

Chairman: F. A. Saez

The Nucleolus—Retrospect and Prospect C. H. Waddington.....	563
--	-----

Appendix

	Page
Report of Nomenclature Committee.....	573
Participants and Addresses.....	575

Indexes

Name Index.....	579
Subject Index.....	589

Introduction ¹

ALEXANDER HOLLAENDER, *Biology Division, Oak Ridge National Laboratory,² Oak Ridge, Tennessee*

The Symposium on The Nucleolus was the fifth in a continuing series of international conferences sponsored by various Latin-American institutions with the encouragement of the Biology Division of the Oak Ridge National Laboratory.

Over a period of quite a few years, a strong cytology group has been developed in Montevideo under the leadership of Clemente Estable at the Institute for Research in Biological Sciences, and its emphasis on the study of the nucleolus made Montevideo a logical place to convene this Symposium. Also, the advances in electron microscopy, new information on the biosynthesis of nucleic acids and proteins, and many other new developments made the structure and function of the nucleolus a very timely symposium subject. The results at the conference clearly justified the decisions of the organizing committee in selecting session topics.

The program was made up during a visit by Dr. Francisco A. Saez of the Montevideo Institute to the United States. As representative of Latin-American members of the organizing committee, he met with Drs. Mary Esther Gaulden,³ Oscar L. Miller, Jr., and R. C. von Borstel of the Biology Division, Oak Ridge National Laboratory; Dr. Robert P. Perry, Institute for Cancer Research, Philadelphia, Pennsylvania; and Dr. Walter S. Vincent, University of Pittsburgh Medical School, Pittsburgh, Pennsylvania. This group worked as a team and received excellent cooperation from many scientists all over the world, but the major responsibility for the organization was carried by Dr. Miller and Dr. Vincent.

The opening session of the Symposium included welcoming addresses by Dr. Estable and Dr. Washington Buño, Dean of the Faculty of Medicine, Montevideo, and a talk on perspectives for the conference by Dr. Jack Schultz of the Institute for Cancer Research, Philadelphia. All papers given at this Symposium and most of the discussion are reproduced in this volume.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Operated by the Union Carbide Corporation for the U.S. Atomic Energy Commission.

³ Present address: The University of Texas, Southwestern Medical School, Dallas, Texas.

It has been interesting to see the development from the first Symposium, in which very few Latin Americans participated, to this fifth one where they played a predominant role. More than eighty established scientists came from various Latin-American countries to attend and take part at Montevideo. In addition, many young Latin-American scientists and a considerable number of medical and basic science students were attracted to the meeting. This enthusiastic participation indicates an increasing emphasis on modern cytology and molecular biology in Latin America, and has more than fulfilled our hope that these Symposia could encourage and bring to the forefront the many interesting developments in basic biological sciences which are now taking place in Latin America. It also indicates that these Symposia are advancing to the status of a very important and permanent activity in the international scientific community.

Special thanks should be given to the local Uruguayan committee that arranged for the meeting facilities and the excellent free-day excursion to Punta del Este, and for the financial support they provided many Latin Americans during their visit to Montevideo. The Organization of American States, the United States National Science Foundation, and the United States Atomic Energy Commission are especially thanked for their generous support. The National Cancer Institute, Bethesda, Maryland, is to be thanked for publication of this Symposium volume.

* * *

Previous Symposia in this series are as follows:

1961

International Symposium on Tissue Transplantation, Santiago, Viña del Mar and Valparaíso, Chile (Proceedings published in 1962 by the University of Chile Press, Santiago; edited by A. P. Cristoffanini and Gustavo Hoecker; 269 pp).

1962

Symposium on Mammalian Cytogenetics and Related Problems in Radiobiology, São Paulo and Rio de Janeiro, Brazil (published in 1964 by The Macmillan Company, New York, under arrangement with Pergamon Press Ltd., Oxford; edited by C. Pavan, C. Chagas, O. Frota-Pessoa, and L. R. Caldas; 427 pp).

1963

International Symposium on Control of Cell Division and the Induction of Cancer, Lima, Peru, and Cali, Colombia (published in 1964 by the U.S. Department of Health, Education, and Welfare; National Cancer Institute Monograph 14, edited by C. C. Congdon and Pablo Mori-Chavez; 403 pp).

1964

International Symposium on Genes and Chromosomes, Structure and Function, Buenos Aires, Argentina (published in 1965 by the U.S. Department of Health, Education, and Welfare; National Cancer Institute

Monograph 18, edited by J. I. Valencia and Rhoda F. Grell, with the co-operation of Ruby Marie Valencia; 354 pp).

Introducción¹

El simposio sobre el nucleolo fue el quinto de una serie continua de conferencias internacionales auspiciadas por varias instituciones latinoamericanas alentadas por la División de Biología del Laboratorio Nacional de Oak Ridge.²

En el transcurso de algunos años, se ha desarrollado en Montevideo un eficiente grupo de citología bajo el liderato de Clemente Estable, en el Instituto de Investigación de Ciencias Biológicas, y su énfasis en el estudio del nucleolo hizo de Montevideo un lugar lógico para celebrar este simposio. Asimismo, los avances en la microscopía electrónica, los nuevos conocimientos sobre la biosíntesis de ácidos nucleicos y proteínas, y muchos otros adelantos hicieron de la estructura y función del nucleolo una materia muy oportuna para un simposio.

Los resultados de la conferencia claramente justificaron las decisiones del comité organizador al seleccionar los temas de las sesiones.

El programa fue preparado durante la visita del Dr. Francisco A. Sáez, del Instituto de Montevideo, a los Estados Unidos. Como representante de los miembros latinoamericanos del comité organizador, se reunió con los doctores Mary Esther Gaulden,³ Oscar L. Miller, Jr. y R. C. von Borstel, de la División de Biología del Laboratorio Nacional de Oak Ridge; con el Dr. Robert P. Perry, del Instituto de Investigación del Cáncer, Filadelfia, Pennsylvania; y con el Dr. Walter S. Vincent, de la Escuela de Medicina de la Universidad de Pittsburgh, Pittsburgh, Pennsylvania. Este grupo trabajó en equipo y recibió excelente colaboración de científicos de todo el mundo, pero los doctores Miller y Vincent fueron principalmente responsables de la organización.

La sesión inaugural del simposio incluyó discursos de bienvenida por el Dr. Estable y el Dr. Washington Buño, Decano de la Facultad de Medicina, Montevideo, y una charla sobre las perspectivas de la conferencia por el Dr. Jack Schultz, del Instituto de Investigación del Cáncer, Filadelfia. Todos los trabajos presentados en este simposio y la mayor parte de las discusiones están reproducidos en este volumen.

Interesante ha resultado observar el desarrollo desde el primer simposio, en que muy pocos latinoamericanos participaron, hasta el quinto, donde desempeñaron un papel preponderante. Más de ochenta científicos de reconocida reputación vinieron de diversos países latinoamericanos para asistir y tomar parte en el simposio de Montevideo. Además fueron

¹ Presentado en el Simposio Internacional sobre el Nucleolo—Estructura y Función, Montevideo, Uruguay, Diciembre 5-10, 1965.

² Operado por la Union Carbide Corporation para la Comisión de Energía Atómica de los Estados Unidos.

³ Dirección actual: Universidad de Tejas, Southwestern Medical School, Dallas, Texas.

atraídos muchos jóvenes científicos latinoamericanos y un considerable número de estudiantes de medicina y ciencias básicas. Esta participación entusiasta indica el creciente énfasis en citología moderna y biología molecular en Latinoamérica, y ha llenado con creces nuestra esperanza de que estos simposios pudieran estimular y traer a primer término los muchos adelantos interesantes que en ciencias biológicas básicas se efectúan hoy día en Latinoamérica. También indica que estos simposios están alcanzando la categoría de una actividad muy importante y permanente de la comunidad científica internacional.

Agradecimientos especiales merece el comité local uruguayo por organizar las facilidades para las reuniones y la excelente excursión del día libre a Punta del Este, y por el apoyo financiero que proporcionaron a muchos latinoamericanos durante su visita a Montevideo. A la Organización de Estados Americanos, la Fundación Nacional de Ciencias de los Estados Unidos y la Comisión de Energía Atómica de los Estados Unidos agradecemos especialmente su generoso apoyo. Al Instituto Nacional del Cáncer, Bethesda, Maryland, le agradecemos por la publicación de este volumen del simposio.

* * *

Los simposios previos de esta serie son los siguientes:

1961

Simposio Internacional sobre Transplante de Tejidos, Santiago, Viña del Mar y Valparaíso, Chile (Actas publicadas en 1962 por la Imprensa de la Universidad de Chile, Santiago; editadas por A. P. Cristoffanini y Gustavo Hoecker; 269 pp).

1962

Simposio sobre Citogenética de Mamíferos y Problemas Relacionados de Radiobiología, Saõ Paulo y Río de Janeiro, Brasil (publicado en 1964 por The Macmillan Company, Nueva York, bajo acuerdo con Pergamon Press Ltd., Oxford; editado por C. Pavan, C. Chagas, O. Frota-Pessoa y L. R. Caldas; 427 pp).

1963

Simposio Internacional sobre el Control de la División Celular y la Inducción de Cáncer, Lima, Perú y Cali, Colombia (publicado en 1964 por el Departamento de Salud, Educación y Asistencia Social de Estados Unidos; Monografía 14 del Instituto Nacional del Cáncer, editada por C. C. Congdon y Pablo Mori-Chavez; 403 pp).

1964

Simposio Internacional sobre Genes y Cromosomas, Estructura y Función, Buenos Aires, Argentina (publicado en 1965 por el Departamento de Salud, Educación y Asistencia Social de Estados Unidos; Monografía 18 del Instituto Nacional del Cáncer, editada por J. I. Valencia y Rhoda F. Grell, con la colaboración de Ruby Marie Valencia; 354 pp).

PREFACE

This, the first *International Symposium on The Nucleolus, Its Structure and Function*, was a scientific meeting of the highest quality. My personal judgment coincides with the unanimous opinion expressed by fellow participants, and I believe that thoughtful reading of the papers, discussions, and cogent final comments of Professor C. H. Waddington will lead readers of this volume to the same conviction.

A most favorable "spiritual atmosphere" prevailed for the interchange of ideas at this Symposium. Undoubtedly there is nothing more solidary, despite individual controversies and personal differences in temperament, than the international attention to scientific research. Inevitably the truths of science prevail as soon as errors are discovered and corrected. The search for truth is our common goal and constitutes one of the essentially democratic aspects of scientific research.

Pertinent to this introduction is the following brief review of principal stages in the progress of our knowledge concerning the nucleolus:

1. First detailed description of the nucleolus (by Fontana in 1781)
2. Demonstration of differing staining properties for the nucleus and the nucleolus
3. Development of the chromosomal "nucleolar organizer" concept toward solution of the mystery of the disappearance and reappearance of the nucleolus during mitosis
4. Discovery through cytospectrophotometry of a relationship between protein synthesis and the nucleolus and associated heterochromatin
5. Development of new techniques which led to revised concepts concerning structure of the nucleolus, to further knowledge of its ultrastructure, and to discovery of the nucleolonema
6. Use of the ultracentrifuge and autoradiography in important biochemical studies, including the technique of *in vitro* hybridization of nucleic acids
7. Discovery of participation of the nucleolus in biogenesis of cytoplasmic ribosomes
8. The important finding that living organisms whose cells lack nucleoli perish at an early stage of ontogenesis
9. Investigations concerning the possibility that the nucleolus might be a carrier of genes
10. Studies to determine the significance of the nucleolus in pathology

Only in death do structures exist isolated from function. In the living organism, structures are always functional and are immersed in a characteristic *intracellular medium* in which they grow. Therefore, the chemistry of the living cell and that of the dead cell are not equivalent, even though they share many common characteristics. The microscopic examination of structures, especially *in vivo*, goes beyond simple anatomical description. It should be stressed that there is a fringe area in which the problems of the cytologist, the physiologist, and the biochemist overlap. At the atomic level of structures, *stereochemistry* does away with the duality of structural components and chemical composition, but it does not invalidate the supramolecular levels of the structures which cannot be interpreted exclusively in chemical terms. For example, some tissues have identical chemical composition yet differ radically in biological function. Obviously, therefore, a purely chemical explanation is very difficult, if not impossible, for many vital functions that *can* be satisfactorily elucidated by the finding of the correlation among the structures (functional synergy).

Unfortunately, an unwarranted disagreement—a *false opposition*, if you will—may sometimes be noticed between biochemists and cytologists. In most cases, data of one discipline complement those of the other; in some, however, communication is poor and hypothetical concepts may seem to contradict each other. (Let us hasten to say that nothing of this prevailed in this Symposium, which gives further reason to praise the “spiritual atmosphere” in which the problems were discussed.)

In future years, the Symposium will help to clarify such issues and prevent the spread of fallacious disagreements. As one example, a committee report presented here (and published in this volume) recommends uniformity in nomenclature concerning nucleolar structures, and is a fertile approach, even if not completely satisfactory, toward better communication.

Credit for the exemplary organization of this Symposium is due Dr. Alexander Hollaender and his collaborators, especially Dr. O. L. Miller, Jr., Dr. W. S. Vincent, Dr. R. P. Perry, Dr. R. C. von Borstel, and Dr. Mary Esther Gaulden; and to the Latin-American members of the organizing committee, among whom Professor F. A. Saez as vice-president of the Symposium and Dr. M. E. Drets as secretary of the committee must be distinguished for their efficient cooperation.

In conclusion, we must especially and sincerely thank Dr. Hollaender. His enlightened, tenacious, and generous leadership has stimulated the successful and fecund initiative for periodically holding symposia or international colloquia in Latin America on basic subjects in biology. Because of the general excellence of these programs, as was the case of the *International Symposium on The Nucleolus*, the symposia have consistently been attended by scientists of high repute.

CLEMENTE ESTABLE

PREFACIO

El *Primer Symposium Internacional sobre el Nucleolo* (estructura y función) realizado en Montevideo, fue un certamen científico de primera categoría. Mi juicio personal coincide con la opinión unánime. La atenta lectura de los trabajos que integran el presente volumen, las discusiones y los agudos y certeros comentarios finales del Profesor Waddington, conducirán a la misma convicción.

En este Symposium predominó un muy favorable clima espiritual para el intercambio de ideas. Indudablemente no hay nada más solidario, dejando de lado las controversias individuales y diferencias personales de temperamento, que la dedicación internacional a la investigación científica. Es inevitable que las verdades de la ciencia se impongan a medida que los errores se descubren y corrigen. La verdad es un bien común y su búsqueda es nuestro objetivo y constituye uno de los aspectos esencialmente democráticos de la ciencia.

Es pertinente mencionar en esta introducción la siguiente breve enumeración de los principales estadios que ocurrieron en el progreso de nuestro conocimiento sobre el nucleolo.

1. Primera descripción detallada del nucleolo (Fontana, 1781)
2. La demostración de que el núcleo y el nucleolo poseen afinidades tintoriales diferentes
3. El desarrollo del concepto del "organizador nucleolar" cromosómico como una solución al misterio de la desaparición y reaparición del nucleolo durante la mitosis
4. El descubrimiento por medio de la citoespectrofotometría de una relación entre la síntesis proteica, con el nucleolo y la heterocromatina asociadas a éste
5. El desarrollo de nuevas técnicas que condujeron a la revisión de los conceptos sobre la estructura del nucleolo, un mayor avance en el conocimiento de su ultraestructura y al descubrimiento del nucleolonema
6. El uso de la ultracentrífuga y de la autorradiografía en importantes estudios bioquímicos, entre los que está incluida la técnica de hibridación *in vitro* de los ácidos nucleicos
7. El descubrimiento de la participación del nucleolo en la biogénesis de los ribosomas citoplásmicos
8. El importante hallazgo de que los organismos vivos cuyas células carecen de nucleolo perecen en un temprano estadio de la ontogénesis
9. Las investigaciones que indican la posibilidad de que el nucleolo pueda ser un portador de genes
10. Los estudios para determinar el significado del nucleolo en patología

Como se ve en el anterior resumen, la evolución de nuestros conocimientos sobre el nucleolo ha sido acelerada por la cooperación entre las diferentes disciplinas científicas. La investigación de hoy en día se hace de acuerdo a problemas más bien que a amplios temas, y a menudo depende más de la cooperación que del esfuerzo concentrado dentro de una determinada disciplina.

En su esfuerzo conjunto para comprender la vida el citólogo no puede prescindir de la química y el bioquímico no puede prescindir de las estructuras celulares. Con este objetivo, los organizadores de esta reunión sabiamente incluyeron ambos aspectos: el estructural y el bioquímico. Por esta razón, creo que este Symposium jalonará un nuevo y significativo estadio en el avance del conocimiento sobre el nucleolo y que este volumen será considerado como un clásico trabajo sobre el tema.

Las estructuras existen aisladas de la función solamente después de la muerte. En el organismo vivo las estructuras son siempre funcionales y están inmersas en el característico *medio intracelular* en el cual ellas se desarrollan; en consecuencia, la química de la célula viva y la de la célula muerta no son equivalentes aunque comparten muchas características. El examen microscópico de las estructuras va más allá de la simple descripción anatómica, especialmente si se hace *in vivo*. Se debe destacar que hay un área límite en la cual los problemas del citólogo, del fisiólogo y del bioquímico se superponen. En el nivel atómico, la estereoquímica elimina la dualidad de la estructura y de la composición química, pero no invalida la estructura supramolecular que no puede ser interpretada exclusivamente por medio de términos químicos. Por ejemplo, algunos tejidos tienen idéntica composición química y sin embargo difieren radicalmente por su función biológica. Una interpretación puramente química es muy difícil aunque no imposible para muchas funciones vitales que pueden ser satisfactoriamente esclarecidas correlacionando las estructuras entre sí (sinergia funcional).

Desafortunadamente, un desacuerdo injustificado—*falsa oposición* si se quiere—se percibe algunas veces entre bioquímicos y citólogos. En muchos casos, los datos de una disciplina complementan los de la otra, en otras sin embargo, la comprensión es pobre y las hipótesis parecen contradecirse entre sí. Apresurémonos a decir que nada de esto sucedió en este Symposium, lo que nos da más motivo para elogiar el clima espiritual en que los problemas fueron discutidos.

Este Symposium ayudará a esclarecer estos problemas y a impedir la extensión de desacuerdos falaces. El informe del comité que se publica en este volumen recomienda una nomenclatura uniforme para las estructuras nucleolares. Esto es un ejemplo de una fértil tentativa aunque no completamente satisfactoria hacia una mejor comprensión.

El mérito de la ejemplar organización de este Symposium corresponde al Dr. Alexander Hollaender y sus colaboradores, especialmente Dr. O. L. Miller, Jr., Dr. W. S. Vincent, Dr. R. P. Perry, Dr. R. C. von Borstel, y Dr. Mary Esther Gaulden y a los miembros latinoamericanos del comité

organizador, entre los cuales se deben destacar al Profesor F. A. Saez vice-presidente del Symposium y al Dr. M. E. Drets como secretario del comité por su eficiente cooperación.

Como corolario debemos agradecer especial y sinceramente al Dr. Hol-laender. Su luminosa, tenaz y generosa rectoría impulsó la exitosa y fecunda iniciativa para realizar periódicamente Symposios o coloquios en Latino América sobre temas básicos de la Biología. La excelencia de los programas de trabajo es causa de que, como en el caso del Symposium Internacional sobre el Nucleolo, concurran los investigadores de más prestigio.

CLEMENTE ESTABLE

Perspectives for the Conference on the Nucleolus^{1, 2}

JACK SCHULTZ, *Chairman, Division of Biology, The Institute for Cancer Research, Philadelphia, Pennsylvania 19111*

HABIENDO tenido el placer de asistir a la conferencia anterior de esta serie, sé por experiencia que es un gran privilegio ser otra vez invitado a asistir. Habitualmente uno viene esperando ser estimulado por la excitación que rutinariamente produce la conferencia—y vean!—uno halla las hermosas y brillantes vistas de este extraordinario y bello paisaje de América Latina. Viniendo del norte, en esta estación del año, aún el recuerdo de que el sol existe es un gran premio. Y más aún, realza el placer, la expectativa de volvernos a reunir con nuestros colegas, cuyos intereses y talentos han sido implicados en el tema central de esta conferencia. No creo prematuro expresar, en nombre de todos los participantes, nuestras entusiastas gracias por esta oportunidad.

Now I must lay aside the mask, and unfortunately continue in the language to which I am limited.

What is the nucleolus, and why should we be so interested in it as to have come together? We have known it was there, in the nucleus, since 1781 when Fontana noted its occurrence in a treatise on the venom of vipers. I have never seen this paper and Montgomery quotes it from a reference by Carnoy. Montgomery himself, as a young man at the beginning of his career, published in the 1898 volume of the *Journal of Morphology* a 300-page review of the work that had been done on the nucleolus since Fontana's time. His review has something like 700 references—all of the observations that had been made on the nucleolus in the hundred-odd years since 1781. It was a good classical thesis, the kind of compilation that marks the end of an era. In that time, most of the initial questions had been asked. Was there a constant number of nucleoli per cell? No, there was not. Did nucleoli occur everywhere? Well, practically everywhere. Where were they at their best? In cell types that

¹ Presented at the Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported in part by Public Health Service research grants CA-01613 and CA-06927 from the National Cancer Institute.

were actively growing and, in general, the larger the cell the larger its nucleolus. To arrive at these generalizations, the cytology of all of the phyla from the protozoa on up had been examined, both in animals and in plants. Montgomery made a table in which he tabulated the numbers of nucleoli found in the oocytes of 175 different species. This, in an attempt to determine whether the number of nucleoli is constant.

It is instructive merely to read a list of the topic headings of these general comparisons and conclusions in the context of our own program: The Chemistry of the Nucleolus; The Number of Nucleoli; The Position of the Nucleolus in the Nucleus; The General Morphological Structure of the Nucleolus; The Polarity of the Nucleolus (where do the vacuoles appear?); Amoeboid Movements, Divisions and Fusions of Nucleoli; Paranucleoli and Pseudonucleoli, Double Nucleoli, etc.; The Relation between Nucleoli and Centrosomes; Ontogenetic Origins of the Nucleolus; Discharge of Nucleolar Substance from Resting Nuclei; Behavior of Nuclei during Nuclear Divisions; and finally, The Function of the Nucleolus and the Comparison of the Nucleoli in Plants, Protozoa, and Metazoa. The striking thing is that in this list there is no separate discussion of the relation of the nucleoli to the chromosomes. The reason for this is found in Montgomery's own discussion. The chromosomes and nucleoli are obviously associated in many cases but the nucleolus being so big and the chromosomes so confused, no really proper discussion of the relationship could be made. Montgomery pointed out, as Wilson also did, that the attempt of Carnoy to derive the chromosomes from the nucleoli was obviously an error. In the case he studied, he had confused chromosome nucleoli and the true plasmasomes. These—our true nucleoli—had already been effectively differentiated from the chromosomes by earlier workers, *e.g.*, Strassburger and Zacharias.

By the beginning of the 20th century, the nucleolus had emerged as a nuclear organelle, probably connected in some way with the metabolism of the nucleus, but in *what* way could not be clarified. Related somehow to the mitotic cycle—since it disappeared in the actual mitotic division, in some cases visibly cast out into the cytoplasm, in other cases breaking down quite early in prophase—no general single property could be relied on. The chemical analyses are of particular interest. It was clear that the staining reactions and the solubility properties of the plasmasomes did not resemble those of the chromosomes and, in consequence, names which are better forgotten were given to differentiate substances. It is clear, however, that the observation of the natural history of the nucleolus brought out, in the minds of those shrewd observers, all of the essential problems that in modified form are still being asked today. There was an important exception—namely, the relation of the nucleolus to the chromosomes, and hence to the genetic apparatus.

This early period has a fascinating lesson. The methodology developed by Miescher and his successors for studying the chemistry of the chromosomes depended on the combining of the chromosomal substances with

dyes. On the basis of these methods, Zacharias and others concluded that the nucleoli were, in fact, different substances than those in chromosomes. Notably, Zacharias himself had come very close to the point of view that we now take about the role of the nucleic acids and their relation to growth. Why were such possibilities disregarded for 50 years? Essentially because the conclusions were founded on a methodology which was challenged, and could not provide a firm base thereafter. On the assumption that the basophilia of the material was an evidence of the nucleic acid that it contained, Zacharias could make hypotheses of the kind that he did. But when histochemists came to the conclusion that the combinations of dyes used were not specific, the general scheme was exploded.

What caused the resurgence of interest in the nucleolus after the time when it was merely a structure present in all nuclei, a convenient marker to be used in studying the viscosity of the nucleoplasm by its rate of settling when marine eggs were inverted? I have already noted that the chromosomes, in relation to the nucleolus, were a puzzling matter. In Wenrich's study of the constancy of the chromomere pattern at meiosis in the grasshopper, he noted that the nucleolus was associated with a particular chromosome region. And later, Belar, in his monograph on the chromosome theory of heredity, pointed out that the nucleolus often was associated with the chromosomes but, said he, with a large object of this kind a critical demonstration was very difficult.

It was the imaginative approach of Heitz that cleared the way for further progress. In his studies in the mid-20's, Heitz really was operating in the same tradition as the classical cytologists who unraveled the mitotic apparatus. His reasoning was completely morphological, it was based on the appearances of the nuclei and their relationships to each other. The essence of his analysis was the correlation of the positions of the nucleoli in the nucleus with those of the chromosomes as they appeared in sister nuclei in mitosis. Heitz believed that the nucleoli that he traced through the mitotic cycle originated from regions in the chromosomes, the secondary constrictions, in which the compact DNA characteristic of the metaphase mitotic chromosomes was not present. He called these regions "Sine acid thymonucleinico—SAT," punning on the fact that the constriction caused by these thin places gave rise to what had become known as the "satellites" of the chromosomes; in other words, the SAT chromosomes—the satellited chromosomes—contained regions in which nucleoli had been formed in the earlier stages of the mitotic cycle. And now the thinking about the nucleoli in relation to the chromosomes, already an occasional discussion in terms of the mitotic cycle in the early era, as I have noted, came more into focus and the question whether the nucleolus was really a reservoir of the "matrix" substance of the chromosomes—then a quite popular concept—received a good deal of attention. The odd thing is that at the time he was working at nucleolar function, Heitz was also making an analysis of the chromocenters of plants, what

he called the "heterochromatic regions," and showing that these also were related to specific regions of the chromosomes.

Now this was the time when cytogenetics began really to make its impact on such problems. It would obviously be much better if Dr. McClintock were telling you this story herself. As you know, at that time she was laying the foundations of the cytogenetics of maize by analyzing the chromosome rearrangements that came out of the X-ray experiments. In a decisive piece of work, making use of an appropriate rearrangement, she was able to show that the nucleolus is organized as a specific region of chromosome 6. The "nucleolar organizer" was one of the pyknotic or heterochromatic regions and, when it was broken in this particular rearrangement, both portions could function, albeit to different degrees.

From the point of view that has developed since that time, it is clear that this was effective evidence that the nucleolar organizer is composed of reduplicated regions and that the separation of the rearrangement involved the unequal partition of nucleolar genes. She was able further to relate these findings to the observations of Heitz and point out that the SAT region of Heitz was really the residual place, where the nucleolus has been; and she also at this time considered the experiments of Navashin, pointing out that the differences in the different species with respect to the size of the constriction could be related to the way in which the nucleolus was formed and disappeared, and its size with respect to the contraction of the chromosomes.

These observations, placing the nucleolus as a definite part of the genetic mechanism, transformed the perspective in which its function was to be regarded; prior to this—in the Montgomery era and on the part of the nongenetically oriented cytologists—speculations about the function of the nucleolus had to be placed in the vague area of cell physiology. The definite relationship to the genetic mechanism at once implied that there was present in the chromosome an organelle which was in some way related to the metabolism of the nucleus. Quickly the analysis was generalized from plants to animals. In those days, there was a major plant and it was maize, and there was a major animal and it was *Drosophila*: Kaufmann showed that the nucleolar organizer in the mitotic chromosomes of *Drosophila* was located in the X and the Y chromosomes. When the salivary gland chromosomes became available, by use of rearrangements this localization was further narrowed to a specific place in the giant chromosomes. Also, it became clear that neither SAT regions nor heterochromatic regions were always nucleolar in nature.

It was about this time, in the mid-30's, that I myself became interested in the problems of the nucleolus. I was then working on the variegated strains of *Drosophila*, and had found that changes in gene activity were position effects of heterochromatic regions in rearrangements. The Y chromosome, which contained a nucleolar organizer, has a profound effect on these manifestations. It was an obvious speculation that nucleolar

function might be involved here—possibly by way of a relation of the nucleolus to chromosome structure. This could easily be disproved in any literal form; other factors, not directly related to the nucleolus, could also control gene activity in these rearrangements. One quickly realized the phenomenon was more complex.

By the mid-30's, the old ideas that the nucleic acids had something to do with cell growth had been replaced by the point of view that proteins were the important substances in genes and their specificities. From X-ray analyses of Astbury and the speculations of Dorothy Wrinch, there emerged the beginning of a template hypothesis of synthesis in biological systems. Alexander and Bridges, much earlier, had made analogies to crystal growth.

Attention was refocused on the nucleic acids by the development of ultraviolet microspectrophotometry by Caspersson. His procedure seemed to avoid the pitfalls of earlier cytochemical methods, which had trapped the earlier hypotheses. I myself saw an opportunity of discovering whether the genetic phenomena with which I was concerned could have any relationship to properties of nucleic acid metabolism. Specifically, I made the assumption that the heterochromatic regions might control gene reproduction and activity by playing a dominant role in general nucleic acid synthesis. This was not necessarily the most probable approach to these questions. I remember the late J. B. S. Haldane telling me then that if one wanted to allow the principle of free association to operate in biochemistry, one should consider that contractile processes could be associated with phosphorus-containing compounds, as in muscle and in chromosomes!

When I arrived in Stockholm to study cytological position effects in *Drosophila*, Caspersson was already investigating the relationship of the nucleic acids to the meiotic cycle in the grasshopper. Those of you who have seen the splendid apparatus that now graces his Institute for Cell Research would probably have some difficulty in imagining the prototype of the Universal microspectrograph at which I found Caspersson working. I believe it to be a fact that the laboratory had been used by Berzelius in the early 19th century and we speculated what his reaction would have been to the odd goings-on in his preserves.

Caspersson, to my delight, quickly grasped the possibilities of the genetic material in the exploration of the function of nucleic acids. The function of the nucleic acids had been a preoccupation of the biochemical laboratory under the direction of Einar Hammarsten, who had pioneered in the study of the physical chemistry of DNA, and had just finished his first ultracentrifugal studies with Svedberg. This was the laboratory that had the "best" DNA.

On my way to Stockholm, I had visited with Astbury at Leeds and transmitted his request to Caspersson for material on which he could pursue the preliminary X-ray observations that he had made on the nucleic acids. What followed was the first specific hypothesis of the relation of nucleic

acids to proteins in terms of their geometrical properties, and presaged in a way the complementarity of the double helix that we now know. According to Astbury, nucleotides in the nucleic acids and the amino acids in the polypeptide chains shared a 3.4 Å spacing and, as he envisioned the relation of nucleic acids to proteins in synthesis, the assembly of the protein on the nucleotide chain could have as its complement the assembly of the nucleotides on the polypeptide chain. This hypothesis of Astbury's had important consequences for Caspersson's thinking, as we shall see.

The summer of 1938 was an exciting one. In Zurich the Cytological Congress was followed by the International Physiological Congress, and at these Caspersson and I presented our work on the relation of the nucleic acids to the heterochromatic regions, and on the relation of the cytoplasmic nucleic acids to the growth of salivary glands and ovaries in *Drosophila*. One of the events of that meeting for me was my first encounter with Jean Brachet. It is most unfortunate that he could not join us here. From his earlier studies of the relations of DNA and RNA during embryological development, he had turned to studies of isolated nuclei and the metabolism of the nucleus. We had much to talk about. I had been interested in Brachet's work as a result of the problems encountered in the genetic studies of variegation. Some of the effects already established then indicated that the chromosomes had influences on the cytoplasm of the egg which determined the nature of the behavior of the genes in the embryo. I speculated that the cytoplasm might show differences in the amount of nucleic acids and that the Y chromosome which was responsible for such an effect genetically would be a reasonable system in which to study this. In this context, the interrelations that Brachet had studied between the nucleic acids in the development of the sea urchin were intriguing. But, more important, our finding of a difference in the cytoplasmic nucleic acids, induced by the Y chromosome, reinforced the feeling that the nucleolus—initially quite high on our agenda—required immediate investigation. (Caspersson himself had been much interested in the function of this organelle in the neuron and other mammalian cells he had observed.)

There was not much time—I was already in my second year's leave of absence from the laboratory at Pasadena—and only an exploration could be carried out. It was sufficient, however, to show that the nucleoli contained large amounts of RNA and, just before the war broke out, we surveyed some of the available stocks that had extra Y chromosomes and diverse rearrangements. It was tantalizing to leave the work in so preliminary a state. We found that there were considerable differences between the different strains, and a striking difference between male and female. One wondered whether the Y nucleolus was in fact different from the X nucleolus or whether differences in the metabolism of the two sexes were responsible. The results were published in several papers, but Caspersson was not able to see them before publication since the war had separated us. In these, the data were interpreted to mean that the

two types of nucleic acids, the DNA and RNA, were interrelated; that the nucleolus was an example of the functioning of the genes; and that the nucleolar substance was in fact an immediate gene product.

Meanwhile, Caspersson had been developing his thinking in a different direction. From further analyses of the nucleolus, he had concluded that the proteins in the nucleolus were special, that they were in fact basic proteins which he identified with the classical histones. This detail looms large in current thinking, as we shall hear in the course of the Symposium. But the critical step was to relate this conclusion to Astbury's speculations about protein synthesis and the nucleic acids. Caspersson formulated a scheme, in which the histones were synthesized in connection with the DNA of the nucleus; in turn, the histones promoted the synthesis of RNA which was responsible for the cytoplasmic proteins. The hypothesis now has a quaint flavor, but it contains the essentials of contemporary molecular biology. It happens to be wrong, in the way that some of the thinking of Zacharias and the early workers was wrong, but the core idea is correct. The actual templates are different, however. The proteins are not intermediates, the double helix of DNA provides the complementarity for replication; and for RNA and protein synthesis, one examines the templates in terms of information transfer.

The microspectrophotometric technique met very much the same fate as the earlier staining techniques. It, too, was challenged and it had its weaknesses. These were exposed by the verification, with better and better chemical techniques, of the actual structures of the nucleic acids, their base ratio differences, and the relationships of the absorption spectra to the quantitation of the measurements of substances. Also, and most important, techniques had to be developed which would make statistical treatments of populations of cells profitable within any given individual's lifetime. The largest effort in the intervening years has been spent upon just these problems, with the result that only as of now does the return to the detailed analysis of genetic material seem profitable.

The advances during the war and immediately after in the study of the nucleolus were, as I have noted, of two kinds. They were the further applications, particularly at Columbia, of the microspectrophotometric methods to the use of dyes, the general corroboration of the RNA content of the nucleolus and, in the hands of Brachet and his associates, the development of autoradiographic procedures and their correlation with cell fractionation methods about which Dr. Vincent, who was at Brussels at the time, will have something to tell us. These researches provided the ancestors of the current ribosomal hypotheses. The essential question was whether the cytoplasmic RNA was derived from the nucleus and, specifically, from the nucleolus. These questions have been resolved, but only rather recently, and it is one of the things that we can anticipate hearing about as the Symposium progresses. The point I think still to be emphasized is that, while the general principles have been laid out,

different organisms are so versatile that we should not, from our present vantage point, be unprepared for surprises.

We thus approach the third and modern round of methodology of which this Symposium will present many examples. The basic advances stem from the use of tracers in the study of metabolism, and the use of the microbial organisms in establishing the chemical principles by which the nucleic acids operate. In the bacteria, DNA synthesis is continuous throughout the cell cycle. There is no mitotic apparatus and the "replicon" initiates the formation of the new DNA along the whole of the genetic fiber. There is no visible nucleolus and the synthesis of the RNA proceeds throughout the cycle in response to the conditions of culture.

The information transfer from DNA to RNA to protein is bound up with a different type of structure in eukaryotes. Transcription occurs in the nucleus, translation in the cytoplasm, to make an extreme statement. The nucleolus occupies a special place in this sequence of events. We now know, after the work which will be developed to its utmost boundaries at this Symposium, that the nucleolus serves as source of the ribosomal main-spring of protein synthesis.

We have seen in this discussion how, from the intelligent questions that almost any shrewd and interested observer could put to his organism, we have come by successive approximations to our present state. Now the methods are refined so that special portions of the DNA molecule associated with the synthesis of the specific RNA derived from the nucleolus can be identified, a spectacular accomplishment which Dr. Ritossa will be telling us about and which, in a sense, puts the cap upon the developments of the concept. From the stage in which the modes of occurrence of the nucleolus were established and its rough composition outlined, to the stage in which it was identified as a chromosomal associate, we now progress to the stage in which it is being identified as a specific chromosomal product, and we can look to finding out how nucleolar synthesis is regulated and what the molecular mechanisms are which concern its synthesis.

I should like to close by returning to the problem of the nucleolus in *Drosophila*, which initiated my own interest in this area. It has had rather an odd history. The initial association of the chromosomes with cytoplasmic nucleic acid came about as a result of an apparent dosage effect of the Y chromosome on the amount of RNA in the cytoplasm of the *Drosophila* oocyte. As work progressed, chemical evidence seemed contradictory and put a certain cloud over the microspectrophotometric work. We have been concerned with this contradiction in our laboratory over a period of years and have not felt it proper to leave it hanging. What was involved was again the methodological concerns which are at the heart of essential advances in these fields: the question of identifying the nucleic acids and of being sure that one had an adequate understanding of their behavior. We had pretty well convinced ourselves that the extra Y chromosome appeared not to have any effect, when one measured all of the nucleic acids of the egg. This, of course, could be due to a regulation

or repression of the functioning of the Y nucleolus, of a sort which we will hear about in connection with the work in *Xenopus*. Yet in plants, both in maize and in wheat, Lin and Longwell and Svihla have shown that extra nucleolar organizers make more RNA and, in the case of wheat, more cytoplasmic mass—in other words, more total protein per cell. These divergencies are important and the *Drosophila* material has quite recently thrown some new light on them. Miss Travaglini and I have now found that there are not only comparisons in which the extra nucleolar organizer material appears not to add to the amount of ribosomal RNA, but also equally valid comparisons between different chromosomal systems in which the addition of a nucleolar organizer contributes a significant increase to the amount of ribosomal RNA. These functional peculiarities apparently are correlated with specific chromosomal arrangements of the nucleolar organizers, an idea which it has only recently been possible to entertain seriously, although as a speculation it was implicit in McClintock's original work.

Moreover, with Dr. Francis Ashton, we have made an analysis of the fine structure of the *Drosophila* nucleoli which leads to the conclusion that the nucleolus in this organism is formed from loops extending from the nucleolar organizer. These are wound into secondary coils, thus giving the nucleolus a spherical appearance in the light microscope. Of great additional interest, the Y nucleolus does show different types of loops than the X in the cells of the imaginal discs. Thus, even in the same species, there is indication of differences in nucleolar fine structure correlated with the genetics of the nucleolar organizer.

I have given a personal background in this discussion, which seemed to me relevant for our meeting because it illustrates the metamorphoses of ideas, and the deceptive shapes that old Proteus assumes when grappled with in experiment. We shall hear about ribosomes, and fine structure, and molecular hybrids; we will look forward to analyses of nucleolar genes, and wonder what the operators may be; and finally, we may speculate whether, returning in a hundred or so years, we might find discussions of nucleoli as unintelligible to us as ours would be to Fontana.

NUCLEOLAR STRUCTURE

As with many of its other aspects, the morphology of the nucleolus is very confusing, particularly when studied with light microscopy. The differences in appearance, for example, between an oocyte nucleolus *in vivo* and a fibroblast nucleolus in tissue culture are so great as to make it difficult to believe they could have any common structural or functional basis. In the 12 papers making up this section there is an amazing diversity of organisms examined, yet there is an even more amazing similarity in the conclusions which are drawn with respect to nucleolar structure, no matter what cell may have been studied. The fine structure analyses, leading to the demonstration of nucleolar components or compartments of *fine fibers*, *coarse fibers*, and *granules*, provide a morphological basis with which to interpret the structures demonstrated by light microscopy and histochemistry.

The papers contained in this section encompassed a full day of the Symposium at Montevideo. These sessions were chaired by Professor Gustavo Hoecker, Dr. F. J. S. Lara, Dr. Jack Schultz, and Dr. J. H. Taylor. Dr. J. G. Lafontaine, as an invited participant, was unable to present his contribution in person. It is included, without discussion, in these Proceedings.

Ultrastructural Aspects of the Normal and Pathological Nucleolus in Mammalian Cells¹

W. BERNHARD,² *Institut de Recherches sur le Cancer, Villejuif, Seine, France*

SUMMARY

Various new techniques of ultrastructural cytochemistry, concerning selective staining for DNA- and RNA-containing structures, and digestion techniques with nucleases and proteases have considerably improved our knowledge of the macromolecular organization of the nucleolus. Three main components can thus be identified: 1) RNA-containing fibrils of about 50 Å in diameter and usually predominant in the reticular network; 2) RNA-containing granules of 150 Å in diameter similar to the cytoplasmic ribosomes; 3) a diffuse protein matrix. In addition, threads of nucleohistones connected with the nucleolus-associated outer chromatin wall penetrate deeply into the nucleolar body. Nucleoli from different tissues may have different patterns of distribution of their components. A particularly promising field of nucleolar research is opened with the application of high resolution autoradiography which allows the study of metabolic events at the ultrastructural level. Thymidine incorporation is impor-

tant in the nucleolus-associated outer and inner chromatin of some nucleoli. Uridine incorporation starts on the DNA of nucleolus-associated chromatin and on the fibrillar threads of the nucleolonema. Later, the radioactivity is also found on the granular regions and covers the whole nucleolar body. The fibrillar part may correspond to the newly formed ribosomal RNA, whereas the particulate component may be a special type of ribonucleoprotein granule used for local protein synthesis. Examples of pathologically altered nucleoli from cancer cells and virus-infected material are given, where important changes in the DNA and RNA metabolism occur. Nucleolar segregation can be experimentally produced with actinomycin D, nitroquinoline N-oxide, mitomycin C, aflatoxin, proflavine, and other substances supposed to block nucleolar function by distinct mechanisms. Such experimentally produced lesions may help to understand normal nucleolar function.—*Nat Cancer Inst Monogr* 23: 13–38, 1966.

THE PURPOSE of this paper is to show some normal and pathological variations of the fine structural substrate of the nucleolar organelle. The point of departure will be a brief summary of the present knowledge of electron microscopy of the nucleolus based on conventional techniques. Various reviews of this subject have already been published (1–4). Our

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² I am much indebted to my collaborators Dr. A. Monneron, Dr. E. Suter, and Dr. R. Simard for use of some of their unpublished data referred to in this work. I am also grateful to Miss Françoise Yven for her devoted help in the preparation of this manuscript.

main intention is to show that the exploration of nucleolar fine structure with new methods will bring us much closer to the understanding of its function than could mere descriptive cytology. But such attempts can be successful only if the identification of the biochemical nature of the observed structures becomes possible at the molecular level and if the procedures used allow us to investigate the dynamic metabolic behavior of the observed fine structure. Furthermore, pathological structures will be considered as end results of altered functions. With this idea in mind, some pathological situations presented may help to reveal the relationship between structure and function in the normal cell. In particular this seems possible for the altered nucleoli in virus-infected cells and also for nucleolar lesions produced with certain antimetabolites used for the blockage of nucleic acid or protein synthesis at distinct sites of metabolic pathways. One would expect the nucleolar fine structure to react specifically if the nucleic acid or protein synthesis taking place in this organelle is specifically hit. This, as we shall see, seems to be the case.

THE ULTRASTRUCTURAL MORPHOLOGY OF THE VERTEBRATE CELL NUCLEOLUS AS SHOWN WITH CONVENTIONAL TECHNIQUES

The renewal of interest in nucleolar morphology started with Estable and Sotelo's paper based on light microscopical observation of silver impregnated nucleoli in nerve cells (5). These authors coined the term "nucleolonema" to designate a filamentous, argentophilic structure representing the main part of the nucleolar body, which was found to be embedded in a kind of diffuse matrix, the "pars amorpha." Soon afterward, the first electron microscopic studies on nucleoli (6, 7) showed in thin sections the presence of filamentous elements which formed a loose network or were, on the contrary, condensed into a more or less spherical, spongy body. The resemblance of Estable and Sotelo's light micrographs to the electron micrographs of the early days of ultrastructural research was striking and we did not hesitate later (8) to propose that the same terminology might be applied in both cases, although the very different techniques of fixation did not allow us to conclude that the observed structures were identical. It was already evident that the "nucleolonema" was not an isolated, rolled up, continuous filament but a coalescent network whose meshes sometimes contained a diffuse substance thought to correspond to the pars amorpha. Furthermore, there was no electron microscopic evidence for the continuity of the nucleolar filament during mitosis, as claimed by these authors.

These two components could be recognized in the cells of many vertebrate tissues after simple osmium tetroxide fixation and embedding in methacrylate. On the macromolecular level, both the "nucleolonema" and the pars amorpha were composed of tiny granules and fibrils. Sub-

sequently this terminology was used by many authors, although others carefully avoided it. The existence of the "nucleolonema" was not admitted by various authors (9-11), and the old term "nucleolini" was proposed instead for dense nucleolar inclusions which probably correspond to the same structure in cells fixed and stained differently (10, 11). When the same material as used by the South American authors, similarly fixed and impregnated with silver, was studied in the electron microscope, one did not find a continuous argentophilic filament, but rather a spongy network (12, 13). Some, but not all, elements of this network, in particular the RNA-containing granules, strongly reduced silver after formol-alcohol-uranyl nitrate fixation (13). The fibrillar elements and the diffuse matrix did not reduce silver. But the over-all picture found in thin sections was roughly superposable on the light microscopic observations, and as the term "nucleolonema" had already been so widely used, we thought it wise not to abandon it completely. It can still be used to designate the nucleolar threads forming a network in most vertebrate cells. The term "pars amorpha" is less well defined, since both RNA and nucleolus-associated chromatin may be present in, between, and around the meshes (14, 15).

In the work done when osmium tetroxide was considered the universal fixative, the "nucleolus-associated" chromatin was not seen and therefore was thought to be an artifact of light microscopic techniques (8). Only since the double fixation aldehyde-osmium tetroxide has become a standard fixation procedure has this essential nucleolar component been rediscovered by electron microscopists. There are two reasons for this difference in the preservation of nucleolar chromatin in particular and nucleohistones in general: Osmium tetroxide is a rather poor fixative for this molecular complex, which is partially extracted during fixation or during the subsequent embedding techniques (16). Aldehydes (formalin, acrolein, glutaraldehyde) fix the chromatin much better but also produce a certain degree of shrinkage, especially if classical dehydration procedures are used. Shrinkage is, of course, another artifact but most useful, since the chromatin can be easily localized wherever it occurs in the nucleus as a dense, highly contrasted substance, especially after uranium stain. On the other hand, contraction of the nucleohistone filaments does not seem very pronounced if the techniques are handled carefully.

THE NUCLEOLAR MORPHOLOGY AS REVEALED WITH TECHNIQUES OF ULTRASTRUCTURAL CYTOCHEMISTRY

The era of osmium tetroxide cytology has contributed little to the knowledge of the nuclear fine structure in general, and to nucleolar morphology in particular, compared to the innumerable new facts revealed in the cytoplasm. The situation changed when unconventional procedures allowed cytochemical identification of fine structure. Two different approaches

have been fruitful: 1) The cells are treated with cytochemical reagents in aldehyde-fixed blocks or frozen sections, postfixed or not in osmium, and embedded as usual in hydrophobic plastics (17, 18). 2) The aldehyde-fixed cells are directly embedded in different types of water-soluble plastics allowing certain types of enzymic extraction and differential stains directly on thin sections (15, 16, 19, 20). The results here reported are based entirely on this second type of technique and are summarized below.

The Nucleolus-Associated Chromatin

Nucleohistones form an outer discontinuous wall around the nucleolus but are continuous with the marginated chromatin and often show irregular extensions into the surrounding nucleoplasm. Importantly, the nucleolar body itself, though often close to the nuclear membrane, is never in direct contact with the membrane (21) but is always separated from it by a thin wall of nucleohistones frequently interrupted by canals leading to the pores in the nuclear membrane (figs. 1, 2, and 3). The structure of these nucleohistones may be almost completely amorphous if the shrinkage is extreme (19) but is composed of extended, twisted filaments of 200–300 Å in diameter if this contraction artifact does not occur. The amount of the nucleolus-associated chromatin is extremely high in glandular cells where protein synthesis is the main function. In most pancreas cells, the nucleohistone wall is large and continuous with a few open sectors (figs. 1, 2, and 3). The liver-cell nucleolus has an intermediate position: Its nucleolar-associated chromatin forms a thinner shell, with large interruptions. In various tissue culture cell lines (HeLa, Kb, BHK, BSC), a very small amount of nucleohistones has been found, covering only some isolated sectors of the nucleolar surface. Although some light microscopic studies suggested the presence of Feulgen-positive material within the nucleolus, only the new cytochemical techniques combined with electron microscopy could demonstrate the presence in most nucleoli of chromatin penetrating from the nucleolar surface deeply into the nucleolar body in the form of irregular strips or regularly spaced lamellae (14, 22, 23). The extent of this intranucleolar chromatin varies considerably in different types of cells and may be greatly increased under certain pathological conditions such as virus infection, where it forms a regular pattern (24).

Another point necessary to mention is the relationship of the nucleolus-associated chromatin with perichromatin and interchromatin granules (17, 25, 26). The former have often been seen in contact with the peripheral nucleolar chromatin, both in pancreas and liver cells, but they were not more frequent in this area than the chromatin marginated along the nuclear membrane. Interchromatin granules do not seem to have any direct relationship with the nucleolus, although it was suggested that they may represent ribonucleoprotein (RNP) particles migrating from the nucleolus toward the cytoplasm (27).

The Nucleolar Body

This term is used here for the nucleolus *sensu strictiori*, without the nucleolus-associated outer and inner chromatin. It is composed typically of the "nucleolonema" and the "pars amorpha" and may also contain isolated or multiple vacuoles. But instead of these rather vague morphological terms to designate very dynamic and constantly changing structures, it seems preferable to identify cytochemically the different macromolecular components of the nucleolar bulk. These results are essentially based on the work of Marinozzi (15, 16, 28).

In all nucleoli examined, granules of about 150 Å in diameter exist. These granules may be irregularly distributed, forming homogeneous areas in some parts of the nucleolus and being completely absent in others. Or they may be found evenly scattered throughout the nucleolar network without preferential localization. In the most typical pancreas nucleolus, however, the granules are localized at the peripheral parts of the nucleolonema (fig. 1). Some nucleoli have very few such granules; others are loaded with them. Their staining reactions are identical with those of the cytoplasmic ribosomes, but they are smaller and their shape tends to be more irregular. In aldehyde-fixed tissues, they are partially digested with ribonuclease and disappear completely if the digestion with the nuclease is followed with pepsin. Therefore, these granules contain both RNA and protein. They may be called nucleolar RNP particles.

The fibrillar component of 50–80 Å in diameter which was described in many papers based on osmium fixation is less visible after aldehyde fixation, as it appears to be embedded in a diffuse amorphous matrix, easily removable, however, with pepsin digestion. Then the fibrils are seen throughout the nucleolar bulk, but in most cases they appear concentrated in the threads of the nucleolonema. Their real length is difficult to indicate because of the sectioning technique used, but fibrils up to 500 Å have been measured. These fibrils are very sensitive to ribonuclease digestion, whereas deoxyribonuclease does not remove them. It can therefore be concluded that they contain RNA. In many cases, transitions between fibrils and granules have been observed (15). Probably fibrils are wrapped up into irregular, twisted elements that finally appear as RNP granules. But the details of this process are obscure. *The important fact remains that RNA is present in two distinct morphological forms in nucleoli of vertebrate cells, one granular and the other fibrillar.* The same observations have been made on invertebrate cell nucleoli (28, 29).

Even after prolonged ribonuclease digestion, the nucleolar body still contains a considerable amount of material that no longer has the staining reactions for nucleic acid. The reticular network of the nucleolonema can still be recognized, although it has almost completely lost its contrast. This substance is entirely removed with pepsin. We are therefore dealing with a protein or proteins whose nature has not yet been determined with the techniques we have used. Probably some of them are nonhistone,

basic proteins, complexing with the nucleolar RNA. One would therefore expect them to be extracted easier with trypsin, but for unknown reasons this enzyme does not work on sections embedded in the water-soluble glycolmethacrylate.

ELECTRON MICROSCOPIC EVIDENCE FOR MIGRATION OF NUCLEOLAR SUBSTANCE INTO THE CYTOPLASM

The question of this evidence has been posed many times, and the answer still remains uncertain (30). In various invertebrate cells but also in mammalian oocytes (31, 32) and very rarely in cell lines cultivated *in vitro* (33, 34), total or partial nucleolar extrusion can be demonstrated morphologically. However, no visible transport of material can be detected in most cases, although the nucleolus is frequently found attached to the nuclear membrane, and nuclear pores are often in direct contact with the nucleolar bulk (fig. 1). No visible nucleolar material accumulates on the opposite cytoplasmic side. On the other hand, we have no indication that in the material observed in our laboratory the nucleolar RNP particles migrate as such across the nuclear membrane. If they do go across the pores, they may get transformed into an invisible form. More likely, the fibrils migrate into the cytoplasm (fig. 1a), but as they are so small and lack contrast, the morphological evidence of such a transport mechanism is rarely found.

PERSPECTIVES OF FURTHER TECHNICAL IMPROVEMENT OF ULTRASTRUCTURAL CYTOCHEMISTRY

If the differential staining and enzymic extraction procedures applied on thin sections have already led to a significant progress in the exploration of the nucleus, it has to be recognized that the number of cytochemical reactions which can be successfully carried out on tissues embedded in the hitherto available water-soluble plastics remains limited. The plastic molecules react with the biological substance during polymerization and thus denature to a considerable extent proteins and nucleic acids. In addition, the hydrated biological molecules lose their water completely during embedding in the plastic. Successful attempts have now been made to embed the tissue in a highly hydrated artificial gel, composed of a mixture of water-soluble monomers and containing up to 80% water (35). These soft gels are frozen in liquid nitrogen and cut with an ultramicrotome at low temperature (36). The first results concerning nucleolar fine structure are encouraging (fig. 2). There is less contraction than usual, and enzymic extraction on such hydrated sections is particularly rapid. Gelatin-embedded tissues were also cut under the same conditions (37). Denaturation of biological substance is probably kept at a mini-

mum, and no extraction occurs (fig. 3). If these methods can be further improved, we may hope to explore nucleolar fine structure close to its natural state.

HIGH RESOLUTION AUTORADIOGRAPHY USED FOR THE STUDY OF NUCLEOLAR FUNCTION

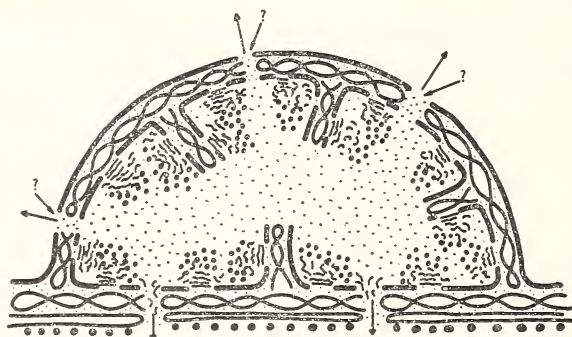
Although numerous autoradiographic studies carried out on the light microscopical level well demonstrated the RNA synthesis of the nucleolus, as well as the migration of nucleolar products into the cytoplasm, nothing was yet known about the precise localization of the sites of incorporation inside the nucleolar apparatus. Refinements of autoradiographic techniques applied to electron microscopy (38) and the introduction of a new sensitive emulsion with very fine grain (39) allowed the study of intranucleolar metabolic events. The few papers published on this subject show the possibilities of this method.

Using tissue cultures from monkey kidney cells labeled with tritiated thymidine, Granboulan and Granboulan (23) could show that a certain number of nucleoli strongly incorporated this precursor not only on the outer wall of the nucleolus-associated chromatin, as it was already noticed on the light microscopical level by Harris (40), but also on the intranucleolar chromatin strips (fig. 4). They were able to demonstrate that other nucleoli, though they contained a considerable amount of nucleohistones, were not labeled. Similar differences were noticed in the light or electron microscope on different cells by Hay and Revel (41). Possibly, the variation of nucleolar DNA synthesis may be linked with the mitotic cycle, as suggested by Harris in his light microscopical studies on synchronized cells (40).

Granboulan and Granboulan (42), using the same techniques, showed the nuclear and nucleolar sites of RNA synthesis in monkey kidney cells.

After a pulse of only 5 minutes, incorporation of uridine can be demonstrated at the peripheral nucleolus-associated chromatin and on its intranucleolar strips. Radioactivity is also found on some parts of the fibrillar nucleolonema. After 10 minutes of uridine incorporation, the number of silver grains is much increased on the fibrillar regions and some are now also found on the granular areas (fig. 5). Simultaneously, uridine incorporation is found throughout the nucleoplasm on the dispersed euchromatin areas but not on the condensed heterochromatin. This observation confirms those made by Littau *et al.* (43) and carried out on isolated nuclei from thymus gland. After 30 minutes of incorporation, the whole nucleolar body is heavily labeled. No preferential localization exists any more. In the euchromatin regions of the nucleoplasm, the picture is the same as after 10 minutes of labeling. As the labeled cells were fixed in aldehydes and embedded in the water-soluble plastic glycolmethacrylate, enzymic digestion could be carried out on thin sections of the same tissue

blocks. The control sections digested with ribonuclease completely lost their radioactivity. In the interpretation of their results, the authors envisage two possible hypotheses: 1) Two distinct types of nucleolar RNA may exist, one with a high, the other with a low turnover. 2) Only a single type of RNA migrates from the fibrillar to the granular part. Although pulse-chase experiments have not been carried out, the second assumption seems more likely because of the observed morphological transition between fibrils and granules (15). In the light of the important biochemical studies of Perry (44-48) showing that the nucleolus produces a high amount of precursor ribosomal RNA, one can tentatively assume that the fibrillar part is the morphological substrate of its 45S precursor (46, 49). This precursor could emigrate to the cytoplasm to form cytoplasmic ribosomes (50) (fig. 1a) or get used locally for the production of nucleolar RNP particles, likely to produce nucleolar or nuclear proteins (text-fig. 1). The RNA synthesized on the dispersed zones of the chromatin could correspond to the messenger RNA, which is not likely to be concentrated within the nucleolus of the mammalian cell as has been suggested for Chironomids (51). The localization of newly synthesized RNA in the fibrillar part of the nucleolus before the granular part was confirmed in a completely different material (*Triturus pyrrhogaster*) by Karasaki (52). With respect to nuclear protein production, it would seem to us incorrect to use the term "nuclear ribosomes" as if there existed a single class of RNP particles. There are at least three types of RNA-carrying granules in the nucleus; the above-mentioned nucleolar RNP particles, the perichromatin granules and the interchromatin granules (15, 17, 25). Their difference in size, localization, staining affinity, and enzymic extraction suggests different functions.



TEXT-FIGURE 1.—Model of a mammalian cell nucleolus, tentatively proposed to interpret its fine structure with respect to its function. Discontinuous outer nucleohistone wall with intranucleolar lamellae. *Twisted filaments*: DNA molecules. *Large, dark line*: histones. Derepressed (*open*) areas synthesize ribosomal RNA fibrils. These fibrils may form RNA particles locally, or emigrate as such into the cytoplasm through the pores of the nuclear membrane. Within the nucleolar body, diffuse protein matrix probably synthesized locally.

SOME EXAMPLES OF PATHOLOGICALLY ALTERED NUCLEOLI

The Nucleolus of the Cancer Cell

Little can be added to the summary presented a few years ago (26). The increased size of the cancer cell nucleolus is a rule with rare exception in classical pathology. Its fine structural variations are numerous and may reach extremes: dense compact nucleolar bodies almost exclusively composed of granules, or, on the contrary, a fibrillar nucleolonema with wide meshes without granules. Vacuoles of all sorts, lipid inclusions, or inclusions of highly contrasted granular material of unknown composition may also be present (53, 54). Not a single lesion hitherto described in the nucleolus can be considered specific for malignancy. Interestingly, some aspects of altered nucleoli in cancer cells resemble those observed after protein deficiency in the rat liver nucleolus (55).

The Nucleolus of the Virus-Infected Cell

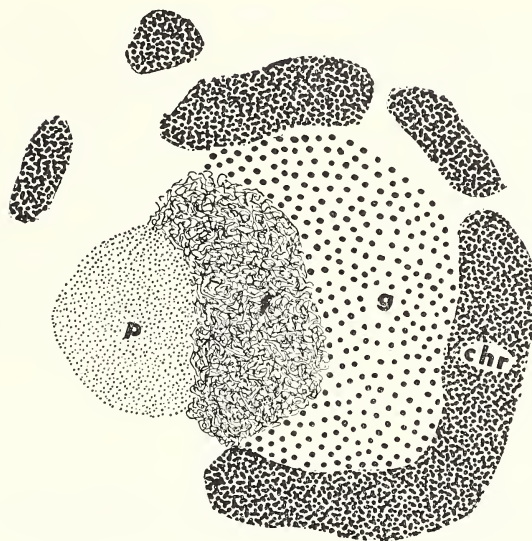
Nucleolar hypertrophy is striking in virus-infected cells and already occurs during the eclipse phase. This hypertrophy may either respect the fine structural architecture of this organelle or completely change it and lead to characteristic lesions. In the RNA viruses developing in the cytoplasm, such lesions are generally more discrete, but also exist. Spotted nucleoli are frequent, however, and difficult to explain in view of the fact that certain types of RNA viruses are thought to develop independently from the nucleus (56, 57). Similar lesions have also been found in cells infected with viruses of the pox group (DNA) developing in the cytoplasm (58, 59). For the whole group of the DNA-nuclear viruses, the nucleolar lesions are always much more pronounced and finally lead to the complete destruction of this organelle, as was shown by many authors. Particular attention has been paid to the nucleolar changes in the SV40 virus, which have been studied both with cytochemical procedures in the light microscope (60, 61) and at the ultrastructural level (24, 62). Besides the general nucleolar hypertrophy in the early hours of infection, profound changes occur in the fine structural organization. The intranucleolar chromatin increases rapidly around the 10th hour and forms a regular lamellar pattern. Later, the RNA fibrils are more numerous and form dense condensations; around the 18th hour many RNP granules become visible. Irregular vacuoles as well as dense granules may also appear. Finally, the nucleolus is completely destroyed and its remnants are dispersed in the nucleoplasm. An attempt at quantitative high resolution autoradiography carried out on the same material gave the following results: There is increased nucleolar DNA synthesis detectable at the 10th hour of infection. At the 18th hour it also increases in the nucleoplasm until the 24th hour, when many infectious virions are already present. The nucleolar RNA synthesis is clearly inhibited at the 10th hour but abnormally high from the 18th hour onward. These changes do not occur

in the corresponding controls. The authors conclude that the nucleolus is important in the synthesis of the SV40 virus and suggest that this organelle may also be involved in the synthesis of other DNA viruses. Quantitative high resolution autoradiography may be a useful tool in the analysis of the eclipse phase of virus infection.

The Phenomenon of "Nucleolar Segregation" Induced With Various Types of Antimetabolites

The first observation on the specific nucleolar action of an antibiotic (actinomycin C) was made with time lapse microcinematography (63). Later, the disruption of the nucleolar body in cells treated with different types of actinomycins was analyzed with phase contrast (64). An electron microscopic study on HeLa cell strains, one resistant, the other susceptible to this antibiotic, was published by Journey and Goldstein (65). The fine structural details on these peculiar nucleolar changes were described as "coalescence," "sorting out," or "redistribution" (66) of the normal nucleolar components or as "nucleolar cap formation" (67). These changes occur not only *in vitro* but also *in vivo* (68). Identical lesions were observed after administration of the skin carcinogen, nitroquinoline N-oxide (69), mitomycin C (70), azaserine (71), and very recently, after injection of very low doses of the newly discovered liver carcinogen aflatoxin in rat liver cells (72). In this latter case, pronounced nucleolar lesions are already visible as early as 30 minutes after the injection, when the rest of the nucleus and the cytoplasm still look perfectly normal. The lesion may therefore appear to be highly specific, although it can be produced with apparently unrelated agents. The term "nucleolar segregation" was proposed for this characteristic type of lesion (72) (fig. 7, text-fig. 2).

According to Simard and Bernhard (73), who were systematically analyzing the possible nucleolar action of many different types of antimetabolites, typical nucleolar segregation also occurs in tissue culture after administration of very small doses (0.2–10 $\mu\text{g/ml}$) of nogalamycin, proflavine, daunomycin, and a few other compounds. Most of the tested products, used in comparable low concentration which, with actinomycin D, lead to immediate segregation, were found negative: aminopterin, mercaptopurine, alkaloids of *Vinca rosea*, different types of purine and pyrimidine analogues, classical alkylating agents, azaguanine, chloramphenicol, puromycin, etc. The agents hitherto found to provoke nucleolar segregation may act somehow on the DNA-RNA template system, whatever their precise biochemical point of attack may be. A possible exception might be azaserine which is thought to block reactions concerning glutamic acid. Among physical agents acting on the nucleolar structure, direct X ray or UV irradiation does not lead to nucleolar segregation (74), but if the nucleolus is shielded and the nucleoplasm irradiated alone, the segregation phenomenon takes place (75). Necrobiosis and autolysis of



TEXT-FIGURE 2.—Schematic representation of a “nucleolar segregation.” A series of antimetabolites induces a redistribution of the nucleolar components, segregated into four different zones: chromatin (chr), RNA granules (g), RNA fibrils (f), and an amorphous protein matrix (p).

cells may lead to a strong densification and partial internal redistribution of the nucleolar components, but do not provoke segregation. Interestingly, nucleolar segregation still occurs in the giant liver nucleoli, produced with thioacetamide (76) (fig. 6), and then briefly treated with actinomycin D or aflatoxin (76).

CONCLUSIONS

The classical electron microscopic techniques based on osmium tetroxide fixation and hydrophobic plastic embedding have favored the exploration of cytoplasmic structures but have contributed relatively little to the knowledge of nuclear and nucleolar fine structure. A series of new, unconventional techniques, allowing cytochemistry and autoradiography at the fine structural level have considerably changed the situation. Further improvement of such methods can be expected. The nucleolus can now be described as an organelle composed of a mixed, but organized, molecular population where different components can be identified: nucleohistones, RNA-fibrils, RNP-granules, and proteins. Although these components are present in all examined vertebrate cells and also seem to be fundamentally similar in invertebrate and plant tissue, their distribution and quantity within the nucleolus vary considerably in different types of cells or even within the same cellular species, depending on the functional state of the

nucleus. These variations are still much more pronounced under pathological conditions, in particular in the malignant or virus-infected cell. In the latter, quantitative high resolution autoradiography allows the exploration of the functional reaction of the nucleus during the developmental cycle of certain viruses. Furthermore, the possibility of experimentally producing specific types of nucleolar fine structural lesions with antimetabolites used in biochemistry for the study of nucleic acid and protein synthesis is expected to narrow the gap between the electron microscopic and biochemical approach. The nucleolus thus appears as a particularly well-chosen model for cell biologists who are interested in the convergence of structure and function at the molecular level.

RESUMEN

Varias nuevas técnicas de citoquímica ultraestructural referentes a tinción selectiva de estructuras que contienen ADN y ARN y técnicas de digestión con nucleasas y proteasas han mejorado considerablemente nuestro conocimiento de la organización macromolecular del nucleolo. Pueden identificarse, de este modo, tres componentes principales: 1) fibrillas que contienen ARN de alrededor de 50 Å de diámetro y predominantes, por lo general, en la red reticular; 2) gránulos que contienen ARN de 150 Å de diámetro muy parecidos a los ribosomas citoplásmicos; 3) una matriz proteica difusa. Además se encuentra que en el cuerpo nucleolar penetran profundamente filamentos de nucleohistonas de la cromatina asociada con el nucleolo. Los nucleolos de diferentes tejidos pueden tener diferentes patrones de distribución de sus componentes. Se abrió un campo particularmente prometedor de investigación nucleolar con la aplicación de la radioautografía de elevada resolución que permite el estudio de acontecimientos metabólicos a nivel ultraestructural. Se ha hallado que la incorporación de timidina es importante en el nucleolo asociado con la cromatina externa e interna de algunos nucleolos. La incorporación de uridina comienza en el ADN del nucleolo asociado con la cromatina, y en los filamentos fibrilares del nucleolonema. Posteriormente, se halla también radioactividad en las regiones granulares y abarca todo el cuerpo nucleolar. Se sugiere que la parte fibrilar puede corresponder al ARN ribosómico recién formado, mientras que los gránulos pueden ser de un tipo especial empleado para la síntesis proteica local. Se dan ejemplos de *nucleolos alterados en forma patológica* de células cancerosas y de material infectado por virus, donde ocurren importantes cambios en el metabolismo del ADN y del ARN.

La *segregación nucleolar* puede ser producida experimentalmente con la Actinomicina D, el óxido -N de Nitroquinolina, la Mitomicina C, la Aflatoxina, la Proflavina y otras sustancias que se supone que bloquean, por distintos mecanismos, la función nucleolar. Se sugiere que dichas lesiones producidas experimentalmente pueden ayudar a comprender la función nucleolar normal.

REFERENCES

- (1) BERNHARD, W.: Ultrastructural aspects of nucleocytoplasmic relationship. *Exp Cell Res Suppl* 6: 17-50, 1958.
- (2) PORTER, K. R.: Problems in the study of nuclear fine structure. *In* Electron Microscopy. Fourth International Congress for Electron Microscopy, Berlin, Sept. 10-17, 1958 (Bargmann, W., Möllenstedt, G., Niehrs, H., Peters, D.,

- Ruska, E., and Wolpers, C., eds.). New York, Academic Press Inc., 1960, vol 2, pp 186-199.
- (3) YASUZUMI, G., SAWODA, T., SUGIHARA, R., KIRIYAMA, M., and SUGIOKA, M.: Electron microscope researches on the ultrastructure of nucleoli in animal tissues. *Z Zellforsch* 48: 10-23, 1958.
 - (4) BUSCH, H., BYVOET, P., and SMETANA, K.: The nucleolus of the cancer cell: A review. *Cancer Res* 23: 313-339, 1963.
 - (5) ESTABLE, C., and SOTELO, J. R.: Una nueva estructura celular: el nucleolonema. *Inst Inv Cien Biol (Montevideo)* 1: 105-126, 1951.
 - (6) BORYSKO, E., and BANG, F. B.: Structure of nucleolus as revealed by electron microscope; preliminary report. *Bull Hopkins Hosp* 89: 468-473, 1951.
 - (7) BERNHARD, W., HAGUENAU, F., and OBERLING, Ch.: L'ultrastructure du nucléole de quelques cellules animales, révélée par le microscope électronique. *Experientia* 8: 58-63, 1952.
 - (8) BERNHARD, W., BAUER, A., GROPP, A., HAGUENAU, F., and OBERLING, Ch.: L'ultrastructure de nucléole de cellules normales et cancéreuses. *Exp Cell Res* 9: 88-100, 1955.
 - (9) BOLOGNARI, A.: Vedute attuali sul nucleolo e sull'ergastoplasma degli ovociti e delle cellule tumorali. *Atti Soc Pelor Sci Fis Mat Nat* 7: 1-104, 1961.
 - (10) BOLOGNARI, A., and DONATO, A.: Particolari aspetti dei "nucleolini." *Caryologia* 16: 439-444, 1963.
 - (11) LOVE, R., and WALSH, R. J.: Studies of the cytochemistry of nucleoproteins. II. Improved staining methods with toluidine blue and ammonium molybdate. *J Histochem Cytochem* 11: 188-196, 1963.
 - (12) GONZALEZ-RAMIREZ, J.: Nuclear physiology. III. Considerations about the meaning of nucleolar silver impregnations in cultured cells. *Bol Inst Estud Med Biol (Mex)* 19: 195-206, 1961.
 - (13) IZARD, J., and BERNHARD, W.: Analyse ultrastructurale de l'argentophilie du nucléole. *J Micr* 1: 421-434, 1962.
 - (14) SMETANA, K., and BUSCH, H.: Studies on the ultrastructure of the nucleoli of the Walker tumor and rat liver. *Cancer Res* 24: 537-557, 1964.
 - (15) MARINOZZI, V.: Cytochimie ultrastructurale du nucléole—RNA et protéines intranucléolaires. *J Ultrastruct Res* 10: 433-456, 1964.
 - (16) ———: The role of fixation in electron staining. *J Roy Micr Soc* 81: 141-154, 1963.
 - (17) SWIFT, H.: Nucleoprotein localization in electron micrographs: metal binding and radioautography. In *The Interpretation of Ultrastructure*, Sympos Int Soc Cell Biol, Berne, 1961 (Harris, R. J. C., ed.). New York, Academic Press Inc., 1962, vol 1, pp 213-232.
 - (18) ———: Cytochemical studies on nuclear fine structure. *Exp Cell Res Suppl* 9: 54-67, 1963.
 - (19) LEDUC, E. H., and BERNHARD, W.: Water soluble embedding media for ultrastructural cytochemistry. Digestion with nucleases and proteinases. In *The Interpretation of Ultrastructure*, Sympos Int Soc Cell Biol, Berne, 1961 (Harris, R. J. C., ed.). New York, Academic Press Inc., 1962, vol 1, pp 21-45.
 - (20) LEDUC, E. H., MARINOZZI, V., and BERNHARD, W.: The use of water soluble glycol methacrylate in ultrastructural cytochemistry. *J Roy Micr Soc* 81: 119-130, 1963.
 - (21) BANNASCH, P., and THOENES, W.: Zum Problem der Nucleolären Stoffabgabe. Elektronenmikroskopische Untersuchungen am Pankreas der weissen Maus. *Z Zellforsch* 67: 674-692, 1965.
 - (22) ALTMANN, H. W., STOCKER, E., and THOENES, W.: Über chromatin und DNS-synthese im Nucleolus. Elektronenmikroskopische Autoradiographische und lichtmikroskopische Untersuchungen an Leberzellen von Ratten. *Z Zellforsch* 59: 116-133, 1963.

- (23) GRANBOULAN, N., and GRANBOULAN, P.: Cytochimie ultrastructurale du nucléole. I. Mise en évidence de chromatine à l'intérieur du nucléole. *Exp Cell Res* 34: 71-87, 1964.
- (24) GRANBOULAN, N., and TOURNIER, P.: Horaire et localisation de la synthèse des acides nucléiques pendant la phase d'éclipse du virus SV 40. *Ann Inst Pasteur* 109: 837-854, 1965.
- (25) WATSON, M. L.: Observations on a granule associated with chromatin in the nuclei of cells of rat and mouse. *J Cell Biol* 13: 162-167, 1962.
- (26) BERNHARD, W., and GRANBOULAN, N.: The fine structure of the cancer cell nucleus. *Exp Cell Res Suppl* 9: 19-53, 1963.
- (27) SMETANA, K.: Unpublished data, 1965.
- (28) MARINOZZI, V., and BERNHARD, W.: Présence dans le nucléole de deux types de ribonucléoprotéines morphologiquement distinctes. *Exp Cell Res* 32: 595-598, 1963.
- (29) STEVENS, B. J.: The effect of actinomycin D on nucleolar and nuclear fine structure in the salivary gland cell of *Chironomus thummi*. *J Ultrastruct Res* 11: 329-353, 1964.
- (30) MAZANEC, K.: Submikroskopische Veränderungen während der Furchung eines Säugetiereies. *Arch Biol* 76: 49-85, 1965.
- (31) IZQUIERDO, L., and VIAL, J. D.: Electron microscope observations on the early development of the rat. *Z Zellforsch* 56: 157-179, 1962.
- (32) SZOLLOSI, D.: Extrusion of nucleoli from pronuclei of the rat. *J Cell Biol* 25: 545-562, 1965.
- (33) GONZALEZ-RAMIREZ, J.: Considerations on nucleolar physiology; the importance of time-lapse cinemicrography. In *Cinemicrography in Cell Biology* (Rose, G. C., ed.). New York, Academic Press Inc., 1963, pp 429-443.
- (34) WEISSENFELS, N.: Struktur und Verhalten der Nukleolen von Hühnerherz-myoblasten in Gewebekultur während des Interphasewachstums und der Mitose. *Z Zellforsch* 62: 667-700, 1964.
- (35) BARTL, P., and BERNHARD, W.: Essais d'inclusion de tissus dans des gels de plastique fortement hydratés. *J Micr* 5: 51-64, 1966.
- (36) BERNHARD, W.: Ultramicrotomie à basse température. *Année Biol* 4: 5-19, 1965.
- (37) BERNHARD, W., and NANCY, M. T.: Coupes à congélation ultrafines de tissu inclus dans la gélatine. *J Micr* 3: 579-588, 1964.
- (38) CARO, L. G., and VAN TUBERGEN, R. P.: High resolution autoradiography. I. Methods. *J Cell Biol* 15: 173-188, 1962.
- (39) GRANBOULAN, P.: Resolving power and sensitivity of a new emulsion electron microscope autoradiography. *J Roy Micr Soc* 81: 165-171, 1963.
- (40) HARRIS, H.: The initiation of deoxyribonucleic acid synthesis in the connective tissue cell with some observations on the function of the nucleolus. *Biochem J* 72: 54-60, 1959.
- (41) HAY, E. D., and REVEL, J. P.: The DNA component of the nucleolus, studied in autoradiographs viewed with the electron microscope. In *Fifth International Congress for Electron Microscopy*, Philadelphia, 1962. New York, Academic Press Inc., 1962, vol 2, p O-7.
- (42) GRANBOULAN, N., and GRANBOULAN, P.: Cytochimie ultrastructurale du nucléole. II. Etude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp Cell Res* 38: 604-619, 1965.
- (43) LITTAU, V. C., ALLFREY, V. G., FRENSTER, J. H., and MIRSKY, A. E.: Active and inactive regions of nuclear chromatin as revealed by electron microscope autoradiography. *Proc Nat Acad Sci USA* 52: 93-100, 1964.
- (44) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (45) ———: Selective effects of actinomycin D on the intracellular distribution of RNA synthesis in tissue culture cells. *Exp Cell Res* 29: 400-406, 1963.

- (46) ———: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat Cancer Inst Monogr* 14: 73–89, 1964.
- (47) ———: Kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA. *J Biophys Biochem Cytol* 11: 1–13, 1961.
- (48) PERRY, R. P., HELL, A., ERREERA, M., and DURWALD, H.: The role of the nucleolus in ribonucleic acid and protein synthesis. I. Incorporation of cytidine into normal and nucleolar inactivated HeLa cells. *Biochim Biophys Acta* 49: 47–57, 1961.
- (49) SCHERRER, K., LATHAM, H., and DARNELL, J. E.: Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 49: 240–248, 1963.
- (50) AMANO, M., and LEBLOND, C. P.: Comparison of the specific activity time curves of ribonucleic acid in chromatin, nucleolus and cytoplasm. *Exp Cell Res* 20: 250–253, 1960.
- (51) SIRLIN, J. L., and JACOB, J.: Function, development and evolution of the nucleolus. *Nature (London)* 195: 114–117, 1962.
- (52) KARASAKI, S.: Electron microscopic examinations of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J Cell Biol* 26: 937–958, 1965.
- (53) HAGUENAU, F.: Significance of ultrastructure in virus-induced tumors. *Nat Cancer Inst Monogr* 4: 211–249, 1960.
- (54) SANKARANARAYANAN, K., and BUSCH, H.: Dense granules in nucleoli of Walker 256 carcinosarcoma cells of the rat. *Exp Cell Res* 38: 434–437, 1965.
- (55) SVOBODA, D., and HIGGINSON, J.: Ultrastructural changes produced by protein and related deficiencies in the rat liver. *Amer J Path* 45: 353–379, 1964.
- (56) FRANKLIN, R. M., and BALTIMORE, D.: Patterns of macromolecular synthesis in normal and virus-infected mammalian cells. *Sympos Quant Biol* 27: 175–198, 1962.
- (57) HINZ, R. N., BARSKI, G., and BERNHARD, W.: An electron microscopic study of the development of encephalomyocarditis (EMC) virus propagated in vitro. *Exp Cell Res* 26: 571–586, 1962.
- (58) DOUBASHKIN, R., and BERNHARD, W.: A study with the electron microscope of the skin tumour of molluscum contagiosum. *J Ultrastruct Res* 3: 11–38, 1959.
- (59) LEUDUC, E. H., and BERNHARD, W.: Electron microscope study of mouse liver infected by ectromelia virus. *J Ultrastruct Res* 6: 466–488, 1962.
- (60) LOVE, R., and FERNANDES, M. V.: Cytological and cytochemical studies of green monkey kidney cells infected in vitro with simian virus 40. *J Cell Biol* 25: 529–543, 1965.
- (61) LEVINTHAL, J. D., and SHEIN, H. M.: Morphology and distribution of SV 40 infected cells in human, simian and hamster renal cell cultures as detected by immunofluorescence. *Proc Soc Exp Biol Med* 112: 405–409, 1963.
- (62) GRANBOULAN, N., TOURNIER, P., WICKER, R., and BERNHARD, W.: An electron microscope study of the development of SV 40 virus. *J Cell Biol* 17: 423–441, 1963.
- (63) ROBINEAUX, R., BUFFE, D., and RIMBAUT, C.: Etude du mode d'action cellulaire de l'actinomycine C. *In La Chimiothérapie des Cancers et des Leucémies. Colloques Int CNRS, Paris, 1957*, pp 225–236.
- (64) BIERLING, R.: Die Wirkung von Actinomycinen auf menschliche Gewebe in vitro. *Z Krebsforsch* 63: 519–522, 1960.
- (65) JOURNEY, L. J., and GOLDSTEIN, M. N.: Electron microscope studies on HeLa cell lines sensitive and resistant to actinomycin D. *Cancer Res* 21: 929–932, 1961.
- (66) SCHOEFL, G. I.: The effect of actinomycin D on the fine structure of the nucleolus. *J Ultrastruct Res* 10: 224–243, 1964.
- (67) REYNOLDS, R. C., MONTGOMERY, P. O., and HUGHES, B.: Nucleolar "caps" produced by actinomycin D. *Cancer Res* 24: 1269–1278, 1964.

- (68) JEZEQUEL, A. M., and BERNHARD, W.: Modifications ultrastructurales du pancreas exocrine de rat sous l'effet de l'actinomycine D. *J Micr* 3: 279-296, 1964.
- (69) REYNOLDS, R. C., MONTGOMERY, P. O., and KARNEY, D. H.: Nucleolar "caps"—a morphologic entity produced by the carcinogen 4-nitroquinoline N-oxide. *Cancer Res* 23: 535-538, 1963.
- (70) LAPIS, K., and BERNHARD, W.: The effect of mitomycin-C on the nucleolar fine structure of KB cells in cell culture. *Cancer Res.* 25: 628-645, 1965.
- (71) HRUBAN, Z., SWIFT, H., and SLESERS, A.: Effect of azaserine on the fine structure of the liver and pancreatic acinar cells. *Cancer Res* 25: 708-723, 1965.
- (72) BERNHARD, W., FRAYSSINET, C., LAFARGE, C., and LE BRETON, E.: Lésions nucléolaires précoces provoquées par l'aflatoxine dans les cellules hépatiques du rat. *C R Acad Sci (Paris)* 261: 1785-1788, 1965.
- (73) SIMARD, R., and BERNHARD, W.: Le phénomène de la ségrégation nucléolaire: spécificité d'action de certains antimétabolites. *Int J Cancer* 1: No. 5, 1966. In press.
- (74) MONTGOMERY, P. O., KARNEY, D., REYNOLDS, R. C., and McCLENDON, D.: Cellular and subcellular effects of ionizing radiations. *Amer J Path* 44: 727-746, 1964.
- (75) MONTGOMERY, P. O., REYNOLDS, R. C., and McCLENDON, D. E.: Nucleolar "caps" produced by ultra-violet flying spot irradiation. *J Cell Biol* 27: 69A, 1965.
- (76) SUTER, E., and SALOMON, J. C.: Effect of l'actinomycine D sur la structure fine du nucléole des hépatocytes de rats intoxiqués par la thioacétamide. *Exp Cell Res.* In press.

PLATE 1

FIGURE 1.—Typical nucleolus of an exocrine pancreas cell of the rat. Fixation: 20 minutes in 2.5% phosphate-buffered glutaraldehyde. Direct embedding in Epon. Combined uranyl acetate-lead stain. Peripheral wall of nucleolus-associated chromatin (chr) composed of twisted fibrillar elements (nucleohistones). Inside, nucleolar body with network of the nucleolonema predominantly composed of tiny RNA-containing fibrils (f). In between, RNP granules (g) smaller than those of the ergastoplasm (er). $\times 82,500$

FIGURE 1a.—Inset representing a high magnification of the area within dotted lines in figure 1. Nuclear pore with many tiny fibrils suggesting migration of fibrillar elements through this pathway. (Electron micrograph made by Dr. A. Monneron.) $\times 180,000$

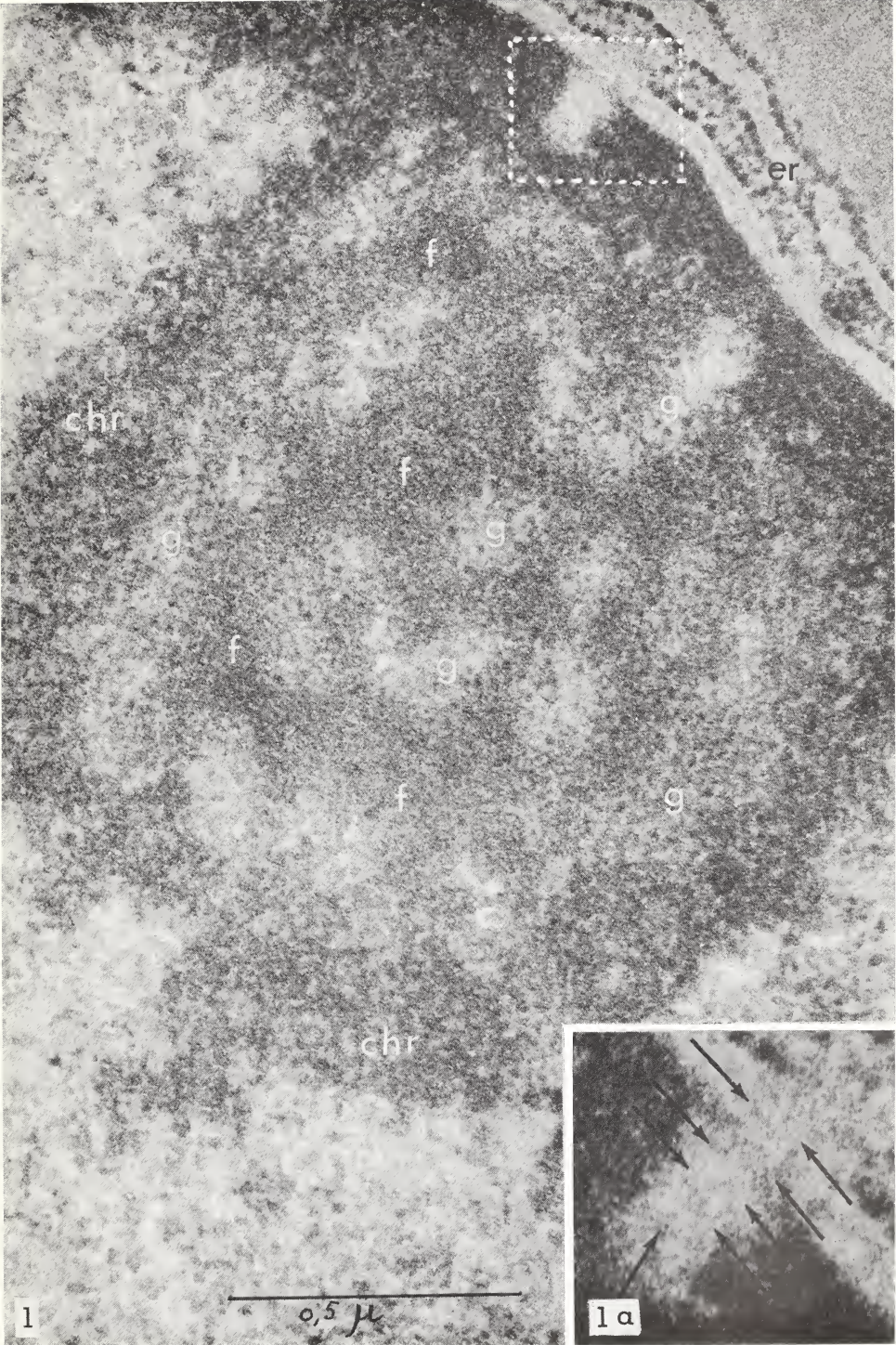


PLATE 2

FIGURE 2.—Nucleolus of an exocrine pancreas cell of the rat. Fixation 1 hour in buffered acrolein-formol solution 5%/5%. Embedded in a gel of water-soluble plastics containing 80% water. Frozen in liquid nitrogen. Cut at -60°C with a freezing ultramicrotome. Uranyl acetate stain. *Note* remarkable preservation of nucleolar structure. Nucleolonema (nu) and associated chromatin (chr). Nuclear pores with canals across the marginated chromatin (*arrows*). $\times 38,000$

FIGURE 3.—Nucleolus of an exocrine pancreas cell of the rat, fixed 1 hour in glutaraldehyde 2.5%. Embedded in 10% gelatin. Frozen in liquid nitrogen. Cut with a freezing ultramicrotome at -60°C . Uranyl acetate stain. Excellent preservation of the ultrastructures which are unusually dense, as no extraction seems to occur. The chromatin (chr) does not take the uranium stain under these conditions. Nucleolonema (nu) ; mitochondria (m). $\times 46,000$

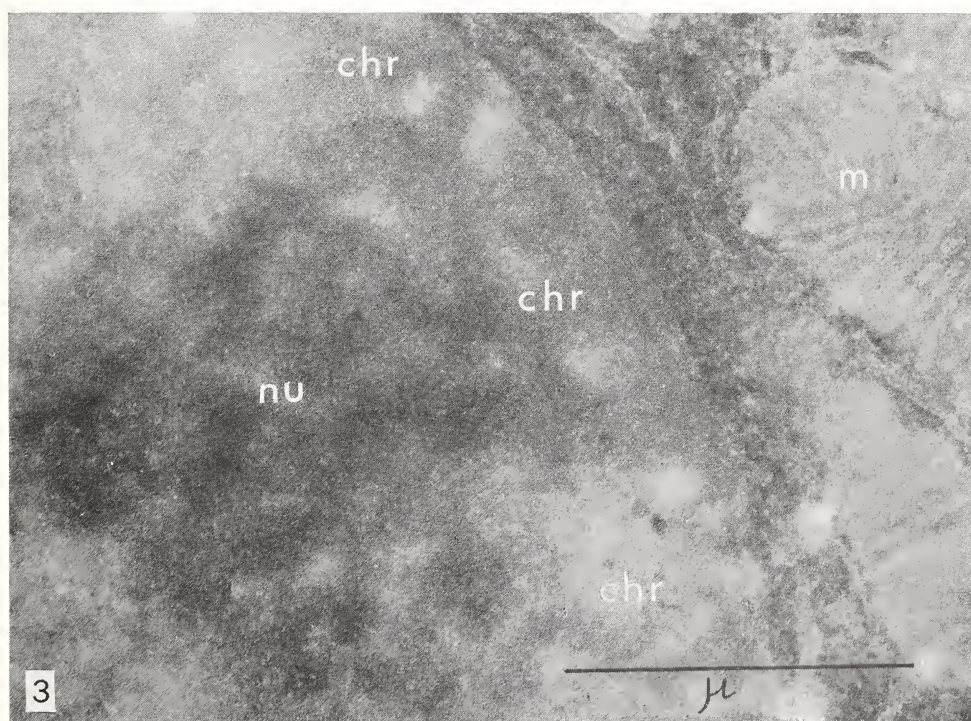
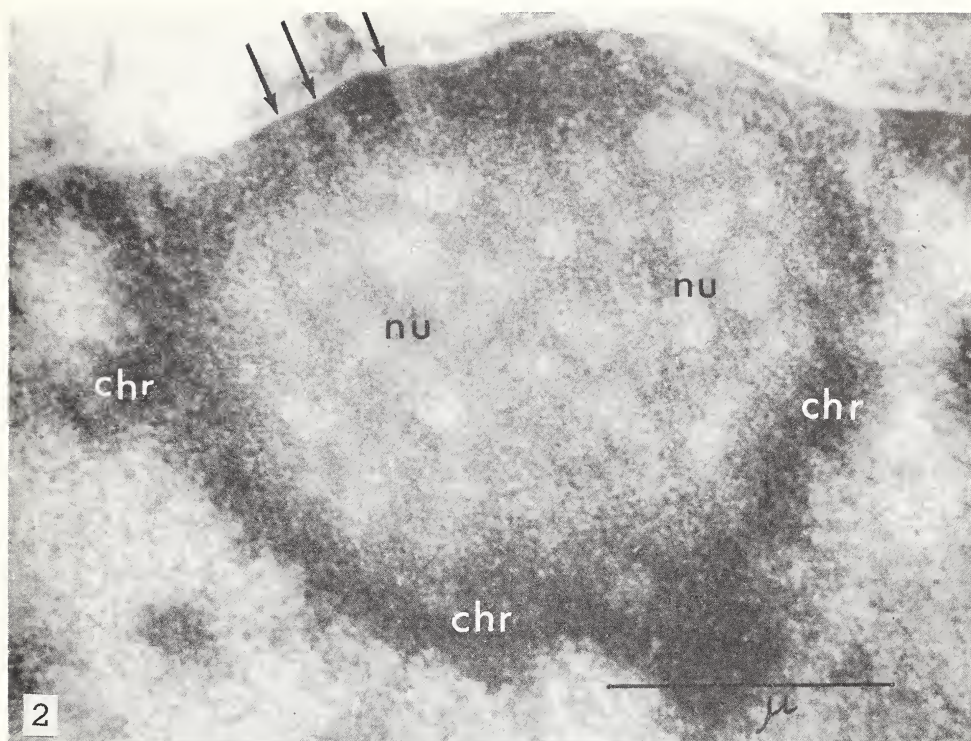


PLATE 3

FIGURE 4.—Nucleolus of a monkey kidney cell in cell culture, labeled for 18 hours with ^3H -thymidine. Formalin-acrolein fixation. Glycolmethacrylate embedding. Treatment with ribonuclease 1 hour. Uranyl acetate stain. Emulsion Gevaert NUC 307. Many bands of intranucleolar chromatin with reduced silver grains visible on some of them. $\times 22,500$

FIGURE 5.—Nucleolus of a monkey kidney cell in cell culture, labeled for 10 minutes with ^3H -uridine. Formalin-acrolein fixation. Glycolmethacrylate embedding. Uranyl acetate. Emulsion Gevaert NUC 307. Localization of labeled RNA in the fibrillar part. Beginning of the passage of labeled RNA in the granular part. (Electron micrographs made by Dr. N. Granboulan.) $\times 32,500$

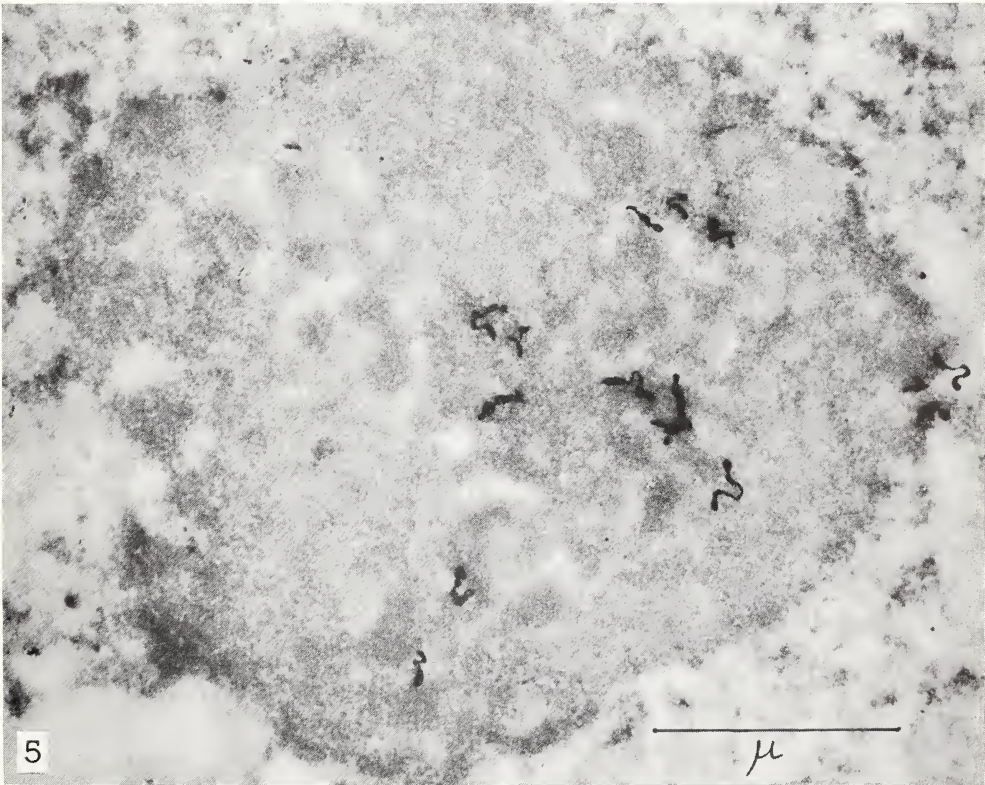
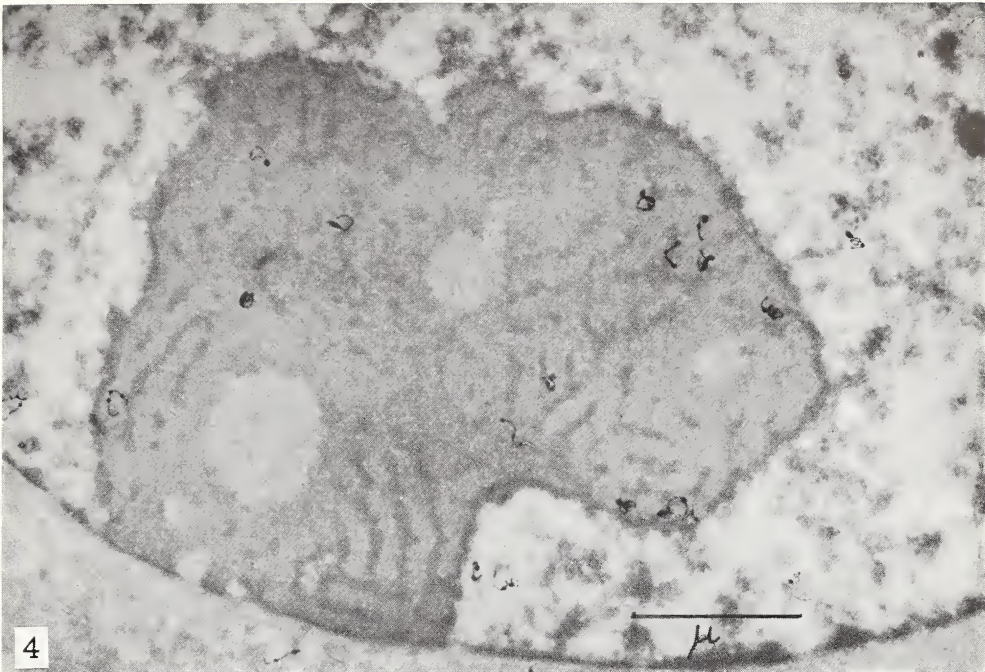
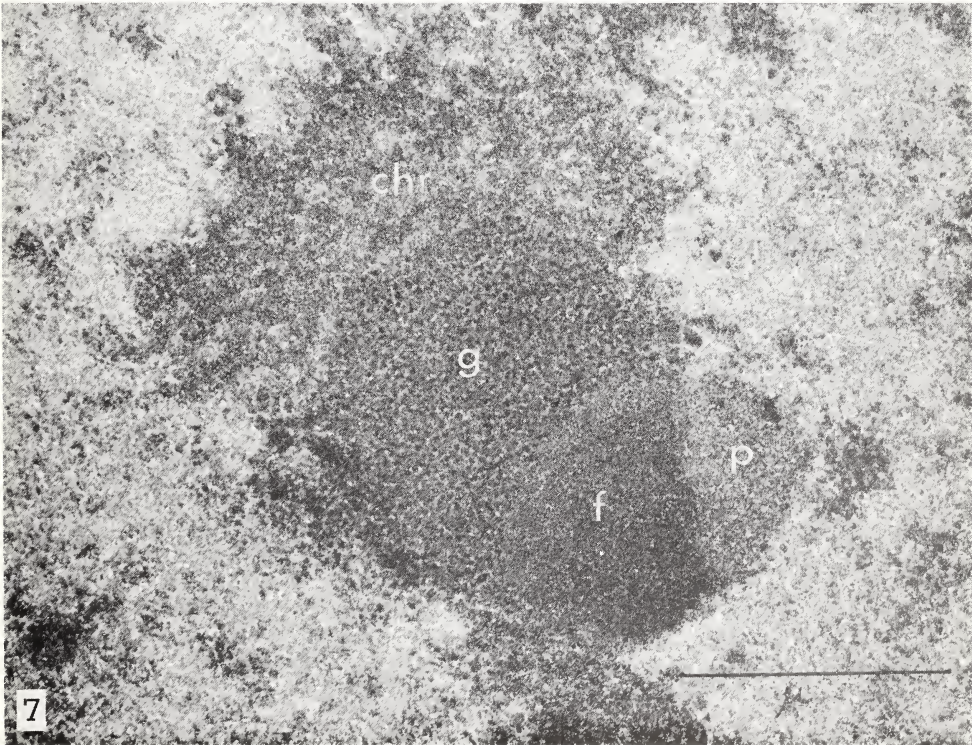
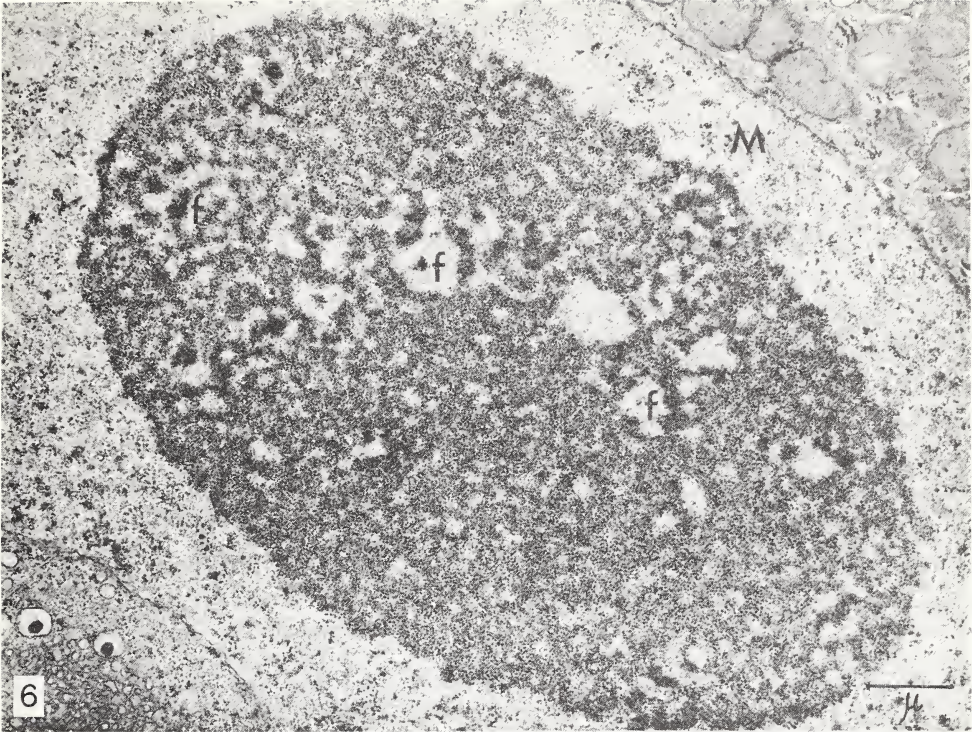


PLATE 4

FIGURE 6.—Giant nucleolus in a rat liver cell from an animal fed 1 month with thioacetamide. Osmium tetroxide. Epon embedding. The nucleolus has only very few fibrils (f) and is almost exclusively composed of granules. Nuclear membrane (M). $\times 11,000$

FIGURE 7.—Nucleolus in a liver cell from a hepatectomized rat, infected with a small dose of aflatoxin. Glutaraldehyde-osmium fixation. Epon embedding. Typical illustration of the phenomenon of "nucleolar segregation." Normal nucleolar components are segregated and redistributed in four distinct zones: proteins (p), fibrillar RNA (f), granular RNA (g), and the nucleolus-associated chromatin (chr). $\times 36,000$



DISCUSSION

Pavan: In the scheme you presented, where is the nucleolonema? In the diagram you showed us, you indicated the existence of a net of DNA distributed inside the nucleolus. This fits very well with our findings in *Rhynchosciara* where the nucleolus is not classical, but is dispersed. Inside this dispersed nucleolus, a network of DNA fibers can be detected. From our results in *Rhynchosciara*, I thought it would be plausible to explain the formation of the nucleolus as the result of a special gene or more probably a special gene complex acting normally such as DNA producing RNA, and this RNA giving rise to certain proteins, and so on. But when I think about Dr. Vincent's statement that 95% of the nucleolus is protein and Dr. Estable's concept that a self-perpetuating system called the nucleolonema exists in the nucleolus, I still cannot visualize a system combining the nucleolonema and a network of DNA, the 5% RNA and the 95% protein giving rise to a nucleolus. If Dr. Bernhard could help me on this, I would be very grateful.

Bernhard: It is difficult to answer you in detail, as you have been studying a system different from ours. The nucleolonema is characteristic for the vertebrate-cell nucleolus. It contains RNA in the form of fibrils and granules as well as proteins. The DNA is at the periphery and in the meshes of the nucleolonemal network. There exist also nucleoli with no nucleolonema but simply an amorphous dense body that may have either fibrillar or granular RNA or both. The questions of terminology will be discussed later by the committee on nomenclature which will publish recommendations.

Sirlin: What is the evidence for the migration of fibrils from the nucleolus?

Bernhard: We have some electron micrographs showing the presence of fine fibrils within the nuclear pores similar to those found in the nucleolonema. I am not absolutely certain that these are RNA fibers, but it seems probable. As yet no author has given convincing evidence that in mammalian cells the RNA crosses the nuclear pores in the form of granules. In Dipteran salivary glands the situation is different, as Dr. Swift has shown.

Sirlin: These fibers are in that region of the membrane where the nucleolus is apposed to it so that they couldn't be coming around from the chromatin?

Bernhard: I don't exclude that RNA formed elsewhere on the chromosomes, e.g., messenger or transfer RNA, may directly pass into the cytoplasm without being shifted to the nucleolus first.

Mandel: I wish to make two remarks. The first one concerns the particles that might leave the nucleoli or nuclei. To determine if the extranuclear ribosomes obtained after incubation of nuclei *in vitro* are attached to the nuclear membrane or are intranuclear in origin, we labeled ribosomal RNA *in vivo* with P^{32} and *in vitro* with adenosine- C^{14} . We found that the polysomes appearing in the extranuclear medium contain both kind of particles. The second remark concerns the nucleolar DNA. In experiments *in vivo* we found that DNA isolated from the nucleolar fraction incorporates various precursors at a much faster rate than chromatin DNA (table 1) (first meeting of the Federation of European Biochemical Soc, 1964, Abstr A 47).

Perry: Could there be a kinetic explanation for the fact that you don't observe any particles migrating from the nucleolus? Since you don't see particles crossing the nuclear membrane, you conclude that such a phenomenon doesn't exist. There could be a kinetic reason for this, namely, that the rate of migration is very rapid compared to the retention. Suppose that the particle is fabricated and retained for

TABLE 1.—Incorporation of H³-thymidine and P³² into liver chromosomal and nucleolar DNA *in vivo* and into ascitic hepatoma cells *in vitro* and *in vivo*

Tissue	Condition	Time	Precursor	Specific activity	
				Chromo- somal DNA	Nucleo- lar DNA
Liver.....	<i>In vivo</i>	2½ hours...	300 µc P ³² /100 g PW.	66.5	1000
"	"	"	"	56.0	3930
"	"	"	"	43.0	3350
"	"	3 hours.....	"	96.0	2200
"	"	3 hours.....	H ³ -thymidine 200 µc/100 g PW	33.4	540
Hepatoma....	<i>In vivo</i>	3 hours.....	300 µc P ³² /100 g PW.	340.0	1320
"	<i>In vitro</i>	30 min.....	10 µc P ³² /ml.....	68.8	5420
"	<i>In vitro</i>	30 min.....	P ³²	115.0	3580
"	<i>In vitro</i>	30 min.....	H ³ -thymidine 1 µc/ml	37.0	7575
"	<i>In vivo</i>	90 min.....	H ³ -thymidine 200 µc/100 g PW	278.0	378
"	<i>In vitro</i>	2 hours.....	H ³ -thymidine 5 µc/ml	470.0	640

Specific activity/cpm/µg DNA P

some time, but once it is released it migrates very quickly. In an essentially steady state situation, one wouldn't see it. I think we should hold this question of particle migration open until we have stronger evidence.

Bernhard: We do not rule out this possibility. However, it is very strange that even when the nucleolus with many granules is in direct contact with a pore, one does not find these granules within the pore or outside, but one does find fibers. Another possible explanation for a different system was given by Dr. Swift. Granules may be transformed within the pores by enzymes and thus get unraveled. The passage across the nuclear membrane may be linked with enzyme systems. The transport of material is unlikely to be simply passive.

Lettré: I am sorry, Dr. Bernhard, but I want to contradict your statement that the nucleolonema you demonstrated in your electron microscopic pictures has nothing to do with DNA, because then we are at the same point where we stood more than 12 years ago. The "nucleolonema," which can be seen in the light microscope and which, under proper conditions, gives a positive Feulgen reaction, disappears in the electron microscopic pictures and vice versa. The "nucleolonema" that you see and that has nothing to do with chromatin disappears in our preparations.

Bernhard: I think that the disagreement is more apparent than real. It all depends on the techniques one is using and the terminology applied. When I said that the DNA has nothing to do with the nucleolonema, I meant that in high resolution electron micrographs no DNA is visible *within* the threads of the nucleolonema. If intranucleolar chromatin is present, it is in between this network. I do not think that the light microscope can distinguish between material found within the nucleolonema or simply attached to it at its periphery. In both cases, one would expect to have a slightly positive Feulgen reaction.

Swift: I would like to make one comment about the presence of fine fibrils within the annulae of the nuclear envelope. Exactly similar fibers may be seen within the

annulae of the annulate lamellae. Since these lamellar structures often occur in the cytoplasm long distances from nuclei, it seems likely that the fine fibrils do not represent a nuclear component seen in the process of passing through the annular pore, but are rather a part of the annulate structure itself, whether it be in annulate lamellae or the nuclear envelope.

Bernhard: The fibers we have observed in the canals of the nuclear pores are not digested with pepsin. Therefore, they do not seem to be proteins.

Changes in Nucleolar Ultrastructure Associated With Differentiation in the Root Apex¹

BEAL B. HYDE, *Department of Botany, University of Texas, Austin, Texas*

SUMMARY

The development of the nucleolus in the root apex of *Plantago ovata* has been studied primarily at the ultrastructural level. Nucleolar volumes in the quiescent center and selected regions of the root cap, cortex, and epidermis correlate qualitatively with cytoplasmic basophilia and mitotic frequency. The *P. ovata* karyotype contains two pairs of chromosomes with terminal nucleolar organizers. The small nucleoli of the quiescent center have been shown by means of serial sections to contain intrusions of heterochromatin into the internal fibrillar zone. The number of intrusions corresponds to what would be expected if each represents the attachment of a nucleolar organizing chromosome. Moreover, the ultrastructural morphology suggests that chromatin is attached to and dispersed in the fibrillar region of nucleoli in interphase cells of all develop-

mental stages. Where cortical and epidermal nucleoli are largest (10–12 times the volume of those in the quiescent zone), the particulate regions are intermingled with the fibrillar regions and the general structure is less compact. Often present is a large central lacuna containing fibrils of a chromatin-like character as well as what appear to be free ribosome-like particles. As division ceases and cells increase in size, the vacuoles occupy an increasing proportion of the cell volume; the nucleoli become more compact and return to a size and configuration like those in the quiescent center. These morphological changes during development suggest that the RNA or ribonucleoprotein of the fibrillar region is made along chromosome fibers and probably gives rise to the ribosome-like particles at the nucleolar periphery.—Nat Cancer Inst Monogr 23: 39–52, 1966.

THE ULTRASTRUCTURE of the nucleolus is well enough described (1–6) to make it practical to ask how it may change during differentiation of a tissue. For this purpose we selected a simple system—a plant root tip—and by means of both light and electron microscopy surveyed changes in the size and ultrastructure of the nucleoli in one of its tissues, the cortex. The observations presented here are part of a larger project to be published in more detail elsewhere.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

The species, *Plantago ovata* Forsk., we selected has several advantages for ultrastructural studies. The primary root tip is small, the meristem being about 1 mm long. Since 2 of the 4 pairs of chromosomes have terminal heterochromatic nucleolar organizers, it has been possible to demonstrate unusually well the ultrastructural relationships between the nucleolus and the chromatin. The chromosomes of *Plantago ovata* are differentiated sharply into heterochromatin and euchromatin (7). Each of the 4 chromosomes bears characteristic regions of heterochromatin on both sides of the centromere. Acetocarmine squashes reveal, particularly in differentiated cells of the *Plantago ovata* root tip, 8 heterochromatic masses at the nuclear periphery. In similar preparations heterochromatic blobs may be seen lying at the periphery of the nucleolus. At the ultrastructure level, heterochromatin and euchromatin will be used to refer simply to regions where the chromatin is compact and diffuse, respectively.

MATERIALS AND METHODS

The material for all work was the primary root meristem of freshly germinated seeds of *Plantago ovata*.

For light microscope studies and measurements, phase contrast photographs were taken of thick sections of Epon-Araldite-embedded meristems prepared as described below.

For electron microscopy, excised root meristems were fixed in collidine-buffered (pH 7.2) 1% OsO₄. All electron micrographs presented here were fixed by this method, although several others, such as cacodylate-buffered 1% OsO₄ and cacodylate-buffered acrolein glutaraldehyde followed by similarly buffered 1% OsO₄, were used for comparative purposes. Tissues were stained with uranyl acetate during dehydration and subsequently poststained with lead citrate after sectioning.

MEASUREMENTS OF NUCLEOLAR VOLUMES

For tissue orientation purposes figure 1 shows the meristem of *P. ovata*. Table 1 presents measurements of nucleoli in several regions of the meristem. All calculations are based on the assumption that the nucleolus is a sphere which, as will be seen, is an approximation. Readings were made on easily identified tissues, where nucleoli were as uniform in size as possible. Because in sections the full diameters of nucleoli might be missed, the smallest nucleoli in a uniform file or group of cells were not measured. In some regions, such as the quiescent zone and inner and outer root cap, very few nucleoli can be measured in any one section. Therefore the data are not to be considered exhaustive.

The volume of the nucleolus increases rapidly as one follows a file of epidermal or cortical initials away from the quiescent zone. It reaches a maximum about one third of the way between the quiescent zone and the

TABLE 1.—Nucleolar volumes in selected tissues of *Plantago ovata* root meristem

Tissue*	Nucleolar volume
Quiescent zone.....	2. 6 μ^3
Epidermis.....	30. 4 μ^3
Largest cortical nucleoli.....	36. 5 μ^3
Inner columella.....	4. 8 μ^3
Outer root cap.....	1. 8 μ^3
Vacuolated cortex.....	10. 0 μ^3

*For location of tissues see figure 1.

beginning of obvious cell enlargement. Beyond the region of maximum volume there is a gradual decrease in nucleolar volume which begins before any obvious cell differentiation.

QUIESCENT ZONE NUCLEOLI

The cells of the quiescent zone with their small nucleoli and low RNA content can be considered as prototypes to the actively dividing and developing cells of this root system. We shall first describe the nucleoli of this region in some detail because nucleoli of all other cells may be thought of as their derivatives. This view finds support in the fact that root tips dissected from seeds soaked only 3 hours, and thus not subject to any considerable growth and differentiation, show nucleoli of the quiescent center type in many tissues. Another main reason for considering these nucleoli first, moreover, is their apparent inactivity, as judged by azure B basophilia. The nucleoli of these cells may be assumed to be of the simplest form and to reveal only essential and ubiquitous components. The larger nucleoli of the active cells may correspondingly have their basic synthetic machinery obscured by the products of synthesis.

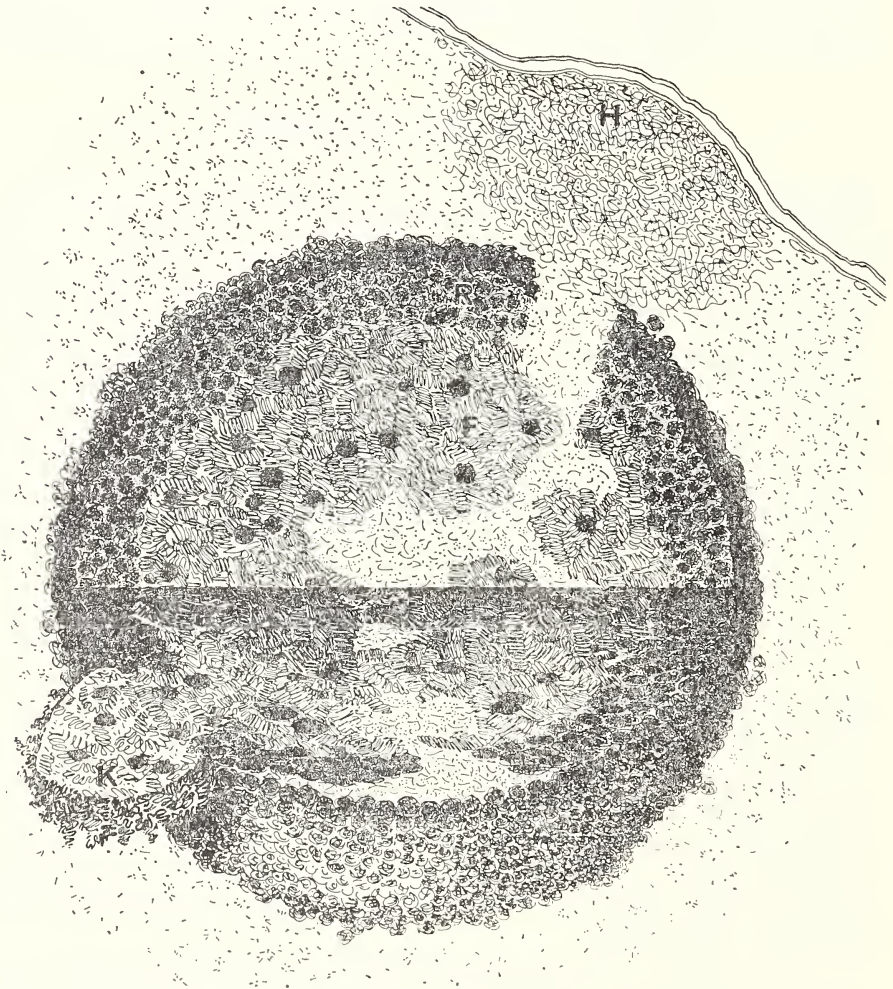
Figure 2 shows a typical nucleolus of the quiescent zone. It resembles the nucleolus of the pea meristem (2) and will be described in the same terms. It contains an internal fibrillar region or pars amorpha characterized by scattered dense granules and a peripheral particulate region. The particulate region will be referred to as ribosomal because particles similar in character to cytoplasmic ribosomes have been prepared from isolated nucleoli (3). Embedded on one side is a karyosome, also characterized by dense granules, but the general texture of which differs from that of the nucleolar interior. This distinction has been discussed in detail elsewhere (2). Heterochromatic masses may be seen appressed to the inner nuclear membrane.

Perhaps the most striking characteristic is that profiles produced by thin sections through most nucleoli of this region are not circular. The outlines, at least in *P. ovata*, are usually like a circle with one or two interruptions occupied by masses of heterochromatin extending toward the nucleolus from the nuclear membrane and bulging into its edge.

THE NUCLEOLUS

The part of the nucleolus in direct contact with these extensions of the heterochromatin is invariably the fibrillar region. As a result of the direct association of heterochromatin with the fibrillar region, the ribosomal cortex is interrupted at the connecting point. Associated with the indentation connecting to heterochromatin, the fibrillar region usually contains one or more light regions having a fibrillar content morphologically identical to the less compact heterochromatic extensions where they are juxtaposed with the nucleolus.

Several series of thick (*ca.* 1100 Å) serial sections through large parts of several nucleoli have made clear two other points which are illustrated in text-figure 1. First, the light regions are not separate chambers but



TEXT-FIGURE 1.—Three-dimensional diagram of nucleolus from the quiescent zone showing relationship between fibrillar region (F), ribosomal region (R), karyosome (K), heterochromatin (H) at nuclear periphery and intrusions of chromatin extending from the heterochromatin. Compare with figure 2.

are always interconnected extensions of the heterochromatin outside the nucleolus. Second, models built from these serial sections, while never including the whole nucleolus, make it seem unlikely that heterochromatin penetrates through the ribosomal cortex through more than four openings. Since *P. ovata* has 2 pairs of chromosomes with terminal heterochromatic nucleolar organizers, it seems reasonable to postulate that these connections between heterochromatin and the fibrillar region represent the attachment of the nucleolar organizer regions.

The karyosome, often seen attached peripherally to the nucleolus, has been described in other plants (2, 9) and is probably a universal component of plant meristem nuclei. Serial sections suggest that sometimes they may lie only slightly embedded in the ribosomal periphery of the nucleoli. But most sections of nucleoli in the quiescent zone which include a karyosome show it to be directly juxtaposed to the internal fibrillar region. Moreover, those karyosomes attached to the fibrillar region frequently share a common opening through the ribosomal periphery with an entering extension of heterochromatin.

CORTEX AND EPIDERMIS

The simplest cases of nucleolar differentiation occur in the epidermis and cortex (defined as the first several layers in from the epidermis), two tissues not significantly different from one another.

For 5–10 cells away from the quiescent zone, there is little change in the appearance of the nucleoli (fig. 3) except for an increase in size. The nucleoli are approximately spherical and the ribosomal layer is peripheral. Regions where the heterochromatin attaches to and penetrates the nucleolus are obvious. Karyosomes may be present, although they are less frequently seen than they are in the quiescent zone and cells adjacent to it. A thin section through a large nucleus and its nucleolus, of course, is a smaller sample of its content than one through a small nucleus. This sampling problem may account for fewer observations of karyosomes as the cells enlarge.

Where the cortical nucleoli are largest, in the region of cell division, they may possess several new characteristics. In material fixed in collidine-buffered 1% OsO₄ the entire structure appears less compact (fig. 4). Both the fibrillar and ribosomal regions are present but they are intermingled. Often, as may also be seen in light microscope preparations, a large central lighter region in the nucleolus can be observed. The nucleolar material immediately surrounding this region is of the ribosomal character. Material within this region has a fibrillar structure like the chromatin outside the nucleolus but, in addition, contains many apparently free particles identical to those in the ribosomal periphery of the nucleolus. These regions, then, are not of the same character as the channels of heterochromatic extensions which are smaller and always inside fibrillar regions.

Much smaller lighter regions inside fibrillar regions surround the large central one. They resemble the heterochromatic channels in nucleoli of the quiescent center, already described.

The looser construction of these largest nucleoli may be seen in both the fibrillar and ribosomal regions. In the fibrillar regions the heterochromatic channels are smaller than in nucleoli of the quiescent zone, probably indicating a more finely branched and more dispersed structure. The ribosomal region appears separated into clumps of particles with some space in between. These clumps may be sections of strands of ribosome-like particles similar to those described by Lafontaine and Chouinard (4) for *Vicia faba* root tip nucleoli or Stevens (10) for nucleoli of *Chortophaga*.

Also in the region of largest nucleoli another characteristic type of nucleolus is found. This type is not spherical but somewhat angular. Often its profile in section is more or less triangular (fig. 5). Serial sections through such a nucleolus indicate that each pseudopod-like extension is the site of a fibrillar region together with an intrusion of heterochromatin. In this type of nucleolus, then, the fibrillar regions associated with the intrusions are largely peripheral, while the interior is filled with ribosomes.

The doughnut-shaped and angular nucleoli are characteristic of the region of the root meristem where mitosis is most frequent. They are not of the late telophase or early interphase nuclei because they are too large. Nevertheless, they have not been associated with any particular stage from late interphase through prophase, since interphase and prophase are difficult to distinguish from each other in an organism such as *P. ovata* which has large masses of condensed chromatin present throughout interphase. Because these two types of nucleoli are common and because division figures are uncommon in the sections we have examined, their characteristics may not be associated with the onset of mitosis.

Along the files of cortical or epidermal cells, basipetal to the region of largest nucleoli, and toward the region of complete vacuolation, the nucleoli change only gradually. The general trend is a reversal of the changes which occurred as the cell developed from the quiescent zone. Therefore, it is appropriate to look at a nucleolus from a highly vacuolated cell 0.7–0.8 mm from the tip. Such a nucleolus (fig. 6) is again compact and small, although not as small as the nucleoli in the quiescent region. Fibrillar and ribosomal regions are present but they differ less from each other than at any previous stage. The large dense granules characterizing the fibrillar region in previous stages of development remain only as vestiges.

The connections of the nucleolus with heterochromatin remain clear, however. These are a persistent and permanent part of all nucleoli observed. Clearly identifiable karyosomes have not been seen associated with these nucleoli. What may have happened to them is not known. Fragmentary observations suggest two possible alternatives: They may fuse with the nucleolus or they may simply disintegrate in the nucleoplasm.

RESUMEN

Se ha estudiado el desarrollo del nucleolo en los ápices radiculares de *Plantago ovata* fundamentalmente a nivel ultraestructural. Los volúmenes nucleolares en el centro quiescente y en regiones seleccionadas de la cofia radicular, la corteza y la epidermis se correlacionan cualitativamente con la basofilia citoplásmica y la frecuencia mitótica.

El cariotipo de *P. ovata* contiene dos pares de cromosomas con organizadores nucleolares terminales. Se ha demostrado mediante cortes seriados que los pequeños nucleolos del centro quiescente contienen intrusiones de heterocromatina en la zona fibrilar interna. El número de intrusiones corresponde al que se aguardaría si cada uno representase la unión de un cromosoma organizador del nucleolo. Además, la morfología ultraestructural sugiere que la cromatina está unida a, y dispersa en, la región fibrilar de los nucleolos en las células interfásicas de todos los estadios del desarrollo.

En donde son mayores los nucleolos corticales y epidérmicos (10–12 veces el volumen de los de la zona quiescente) las regiones ribosómicas se entremezclan con las regiones fibrilares y la estructura general es menos compacta. Con frecuencia está presente una laguna central que contiene fibrillas de carácter semejante a la cromatina, asimismo parece estar libre de partículas semejantes a ribosomas. Cuando cesa la división y las células aumentan de tamaño, las vacuolas ocupan una mayor proporción del volumen celular y los nucleolos se tornan más compactos y retornan a un tamaño y configuración igual a la del centro quiescente.

Estos cambios morfológicos que ocurren durante el desarrollo sugieren que el ARN o la ribonucleoproteína de la región fibrilar se produce a lo largo de las fibras cromosómicas y probablemente origina a los ribosomas en la periferia nucleolar.

REFERENCES

- (1) DAVID, H.: Physiologische und pathologische Modifikationen der submikroskopischen Kernstruktur. III. Der Nucleolus. *Z Forsch* 71: 551–586, 1964.
- (2) HYDE, B. B., SANKARANARYANAN, K., and BIRNSTIEL, M. L.: Observations on fine structure in pea nucleoli in situ and isolated. *J Ultrastruct Res* 12: 652–667, 1965.
- (3) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. IV. The nucleus of *Smittia parthenogenetica* (Chironomidae) with special reference to the nucleolus and the effects of actinomycin thereon. *J Ultrastruct Res* 12: 315–328, 1964.
- (4) LAFONTAINE, J. G., and CHOUINARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167–201, 1963.
- (5) MARINOZZI, V.: Cytochimie ultrastructurale des nucléole-RNA et protéines intranucléolaires. *J Ultrastruct Res* 10: 433–456, 1964.
- (6) SWIFT, H.: Nucleic acids and cell morphology in Dipteran salivary glands. *In* The Molecular Control of Cellular Activity (Allen, J. M., ed.). New York, Toronto, and London, McGraw-Hill Book Co., 1962, pp 73–125.
- (7) HYDE, B. B.: Differentiated chromosomes in *Plantago ovata*. *Amer J Bot* 40: 809–815, 1953.
- (8) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosome. *Biochim Biophys Acta* 76: 454–462, 1963.
- (9) LAFONTAINE, J. B.: A light and electron microscope study of small spherical nuclear bodies in meristematic cells of *Allium cepa*, *Vicia faba* and *Raphanus sativus*. *J Cell Biol* 26: 1–24, 1965.
- (10) STEVENS, B. J.: The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J Cell Biol* 24: 349–368, 1965.

PLATE 5

FIGURE 1.—Detail of apical meristem of *Plantago ovata*. Root tip fixed in collidine-buffered 1% OsO₄ (standard method), embedded in epoxy resin, and photographed with phase contrast optics. Root cap columella (C), root cap (RC), quiescent center (Q), and epidermal initials (E) are indicated.

FIGURE 2.—Typical nucleolus of quiescent center. Distinction between fibrillar region (F) with dense granules, particulate periphery (R), and karyosome (K) is clear. Large intrusion of chromatin is shown. Fixed by standard method.

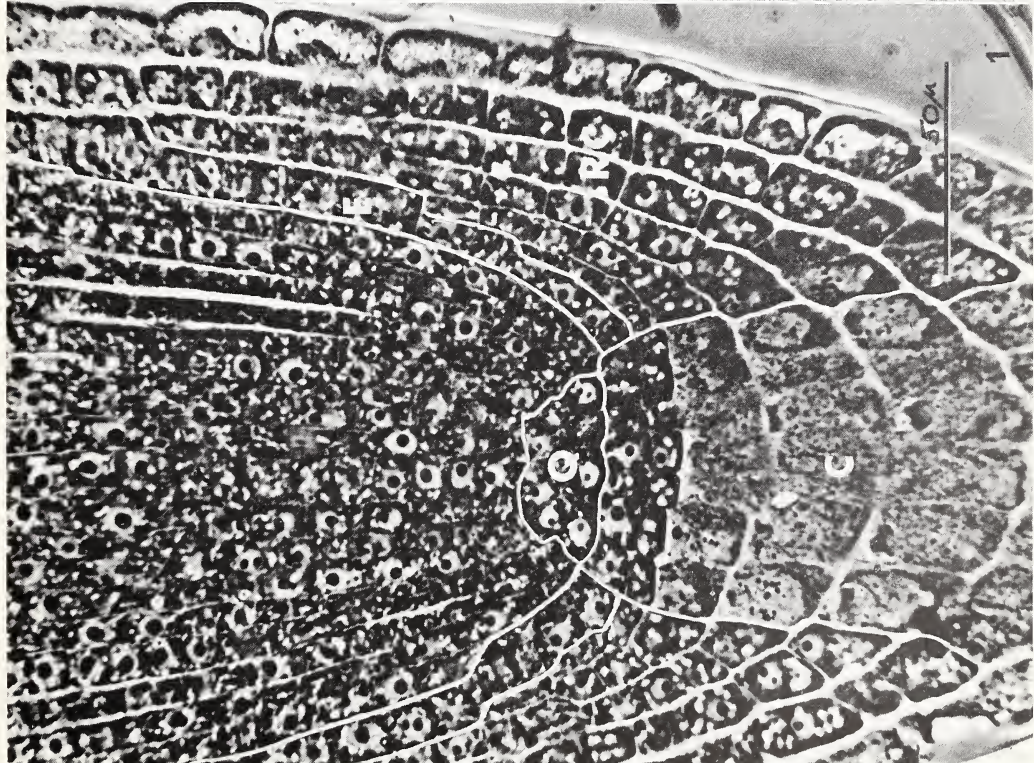
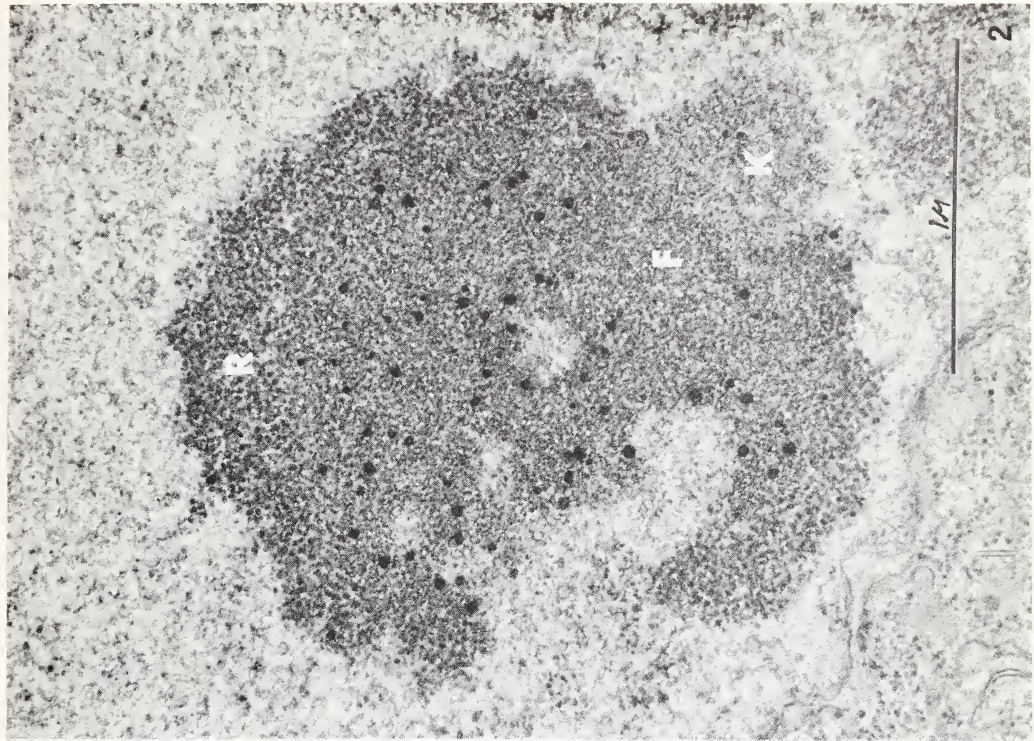


PLATE 6

FIGURE 3.—Nucleolus of young cortical cell typical of nucleoli near the quiescent center in any tissue. *Note* intimate connection of extended heterochromatic fibrils with the fibrillar region of the nucleolus. The ribosomal periphery (R) is looser than in nucleoli of the quiescent center. Fixed by standard method.

FIGURE 4.—Large nucleolus of the cortex where fibrillar and ribosomal regions are intermingled. Large central lacuna appears like chromatin except for high concentration of possibly free ribosome-like particles. Smaller lacunae in fibrillar regions contain only fibrils. Fixed by standard method.

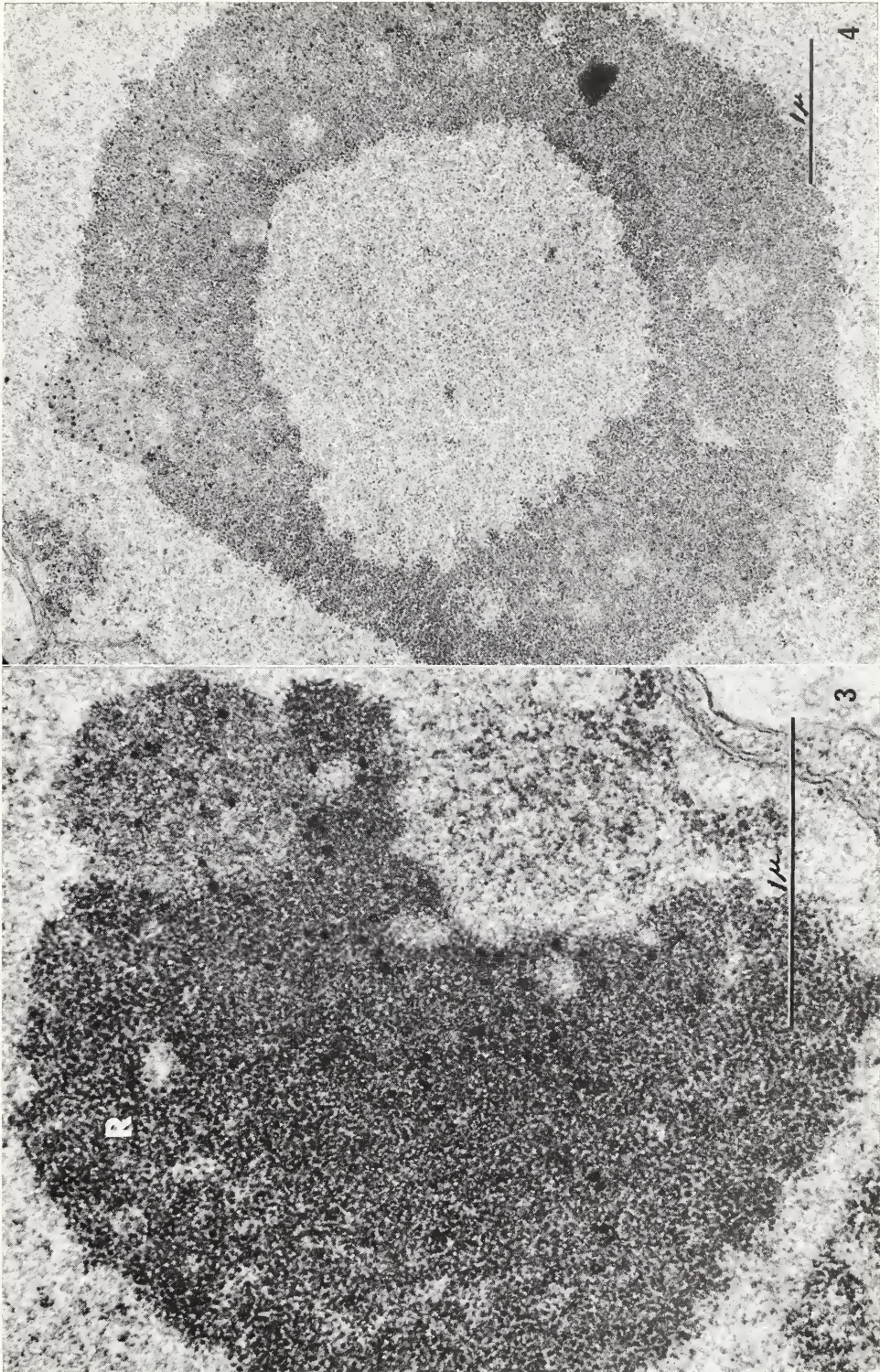
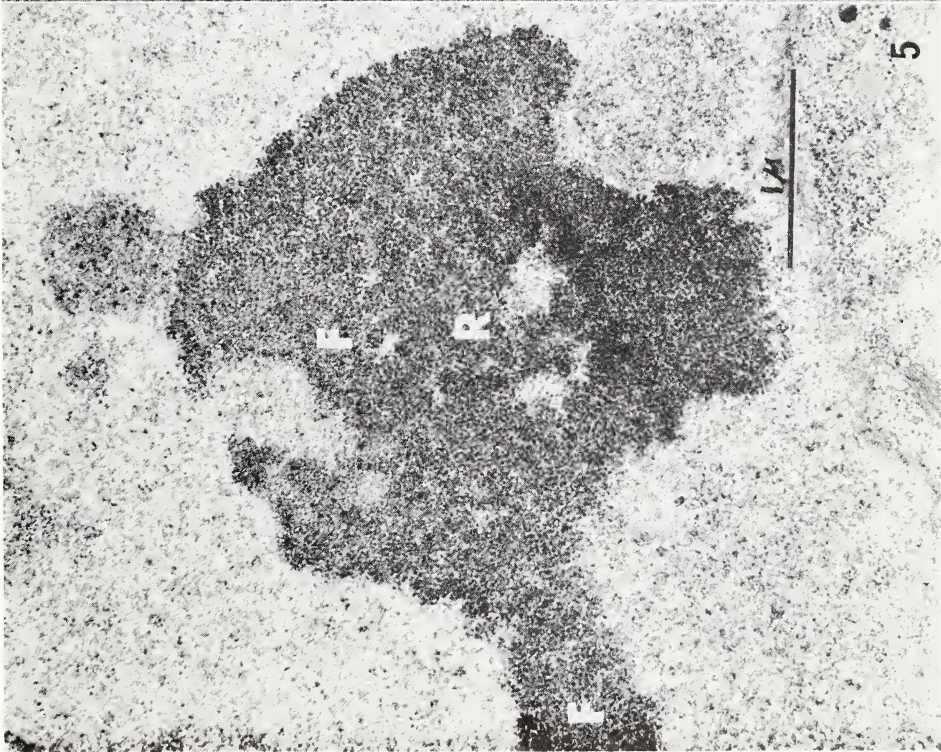
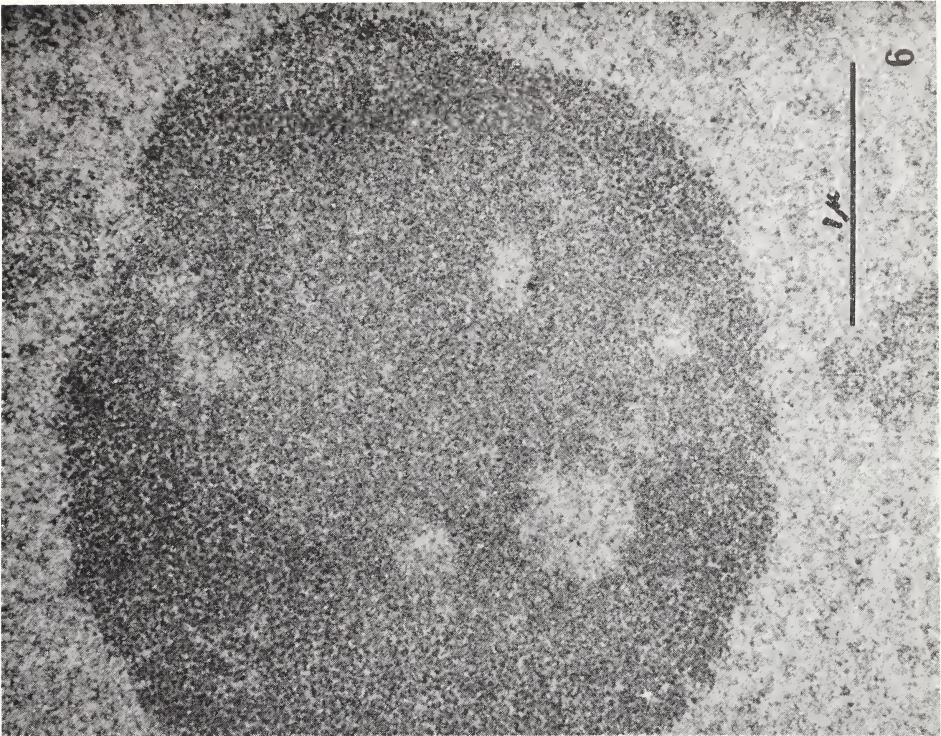


PLATE 7

FIGURE 5.—Irregularly shaped nucleolus of cortex with peripheral fibrillar regions (F) and ribosomal (R) interior. Fixed by standard method.

FIGURE 6.—Nucleolus of highly vacuolated cortical cell 0.7–0.8 mm away from the quiescent center. Fixed by standard method. Configuration of the nucleolus is typical of less differentiated cells near or in the quiescent center. Dense granules of the fibrillar interior are lacking.



DISCUSSION

Taylor: Dr. Hyde, in figure 6 of the paper, could you trace the DNA into the central region? Is that region occupied by DNA at this stage?

Hyde: The nucleolus was from a relatively high vacuolated plant cell, and we did not make serial sections. Consequently, I cannot answer your question.

Barr: Have you ever stained these nucleoli for light microscopy and observed whether the vacuoles are Feulgen-positive?

Hyde: I have only used the staining procedure Dr. Love and Dr. Chouinard have used (Chouinard, *Canad J Bot* 42: 779-785, 1964). Both types of vacuole—the type in the various larger nucleoli as well as the one in the fibrillar region—stain exactly as they say they do, purple surrounded by a cabbage green nucleolus.

Barr: Would you intend to generalize, then, that what are usually called vacuoles in nucleoli are DNA?

Hyde: I would say that it certainly is true in this particular plant.

Pavan: How are you using the term, "karyosome"?

Hyde: I'm very sorry about the word "karyosome," because I'm in considerable awe of the nomenclature committee. In a paper I published I used the term "karyosome" to refer to a structure, usually associated with the nucleolus, which has an internal structure very similar to the fibrillar region of the nucleolus but that is always somewhat distinct, as you saw in my electron micrographs. Moreover, enzymatic digestion suggests that it contains more DNA than the fibrillar region of the nucleolus. I tend to regard the karyosome as a supplementary kind of puff. It may be located somewhere else in the chromosome complement, but works in association with the nucleolus.

Saez: Why do you call them vacuoles, knowing that there is a chromatinic substance contained within these areas of the nucleolar mass? I think that calling them vacuoles makes no sense. The word vacuole means nothing. The nomenclature should be changed and the nomenclature of the nucleolus is one of the points we are discussing at this Symposium.

Hyde: I think it would be elegant if we could get rid of the word vacuole. Maggio has called it a lacuna. We ought to have a term which really says something about what we think it is, perhaps a chromatin structure of some sort.

Structure and Composition of Peripheral Nucleoli of Salamander Oocytes^{1, 2}

O. L. MILLER, Jr., *Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee*

SUMMARY

The nucleus of a medium-sized *Triturus* oocyte contains several hundred peripherally located nucleoli unattached to the chromosomes. In thin sections each nucleolus exhibits a fibrous core surrounded by a granular cortex. In older oocytes, these nucleoli fragment into clusters of smaller spheres, each retaining the bipartite morphology of the parent nucleolus. When nucleoli of either stage are isolated into less than 0.025 M saline, the granular component rapidly disperses. Under these conditions, the nucleolar cores from the medium-sized oocytes expand into loose fibrous networks, whereas those from the nucleolar clusters appear as circles resembling loosely beaded necklaces of various sizes. Treatment of the cores with trypsin or ribonuclease decreases their size, but no breaks occur in the continuity of fibers or circles. Deoxyribonuclease digestion results in the disintegration of cores. Electron microscopy of isolated cores shows that the continuity of the fibrous networks and the interbead strands of the necklaces is maintained by a fiber ~ 30 Å in diameter.

When the granular nucleolar component is dispersed by more gentle methods a structure of low phase contrast can be observed attached to the nuclear envelope side of each core. Depending on the oocyte stage or isolation medium used, this component may assume a spherical shape or appear as a thin filament up to $25\ \mu$ in length. With electron microscopy, these filaments appear as flattened membranous tubules ranging from 500 Å to $0.5\ \mu$ in width and usually contain irregularly spaced granules up to 1000 Å in diameter. Evidence from other studies shows that the first incorporation of RNA precursors occurs at a site in the nucleolus which is coincident with the attachment point of this membranous component and that the incorporation probably is in ribosomal RNA precursor molecules. It is suggested that the membranous nucleolar component may play a role in an intranucleolar transfer or transformation of the ribosomal RNA precursor molecules following their transcription on the DNA in the nucleolar cores.—*Nat Cancer Inst Monogr* 23: 53–66, 1966.

¹ Presented at the Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Research sponsored by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

ONE OF the largest cell types known, the amphibian oocyte, contains a correspondingly large nucleus, ranging up to 1 mm in diameter in some species. Because of the large size of cell and nucleus, individual nuclei and their contents can be isolated by relatively gentle manual methods. In addition, each amphibian oocyte nucleus contains several hundred large nucleoli, which are not physically attached to the meiotic lampbrush chromosomes of this cell. This combination of factors provides a system in which the effects of various chemicals and enzymes on isolated nucleoli can be observed before extensive denaturation occurs. This report describes the results of attempts to characterize the composition and structure of nucleoli isolated from salamander oocytes.

MATERIALS AND METHODS

Oocytes were obtained from *Triturus viridescens*, the common spotted newt of eastern North America, and *Triturus pyrrhogaster*, a salamander of Japan. In order to compare isolated nucleoli with those in intact cells, sections for electron microscopy were prepared by the usual procedures of buffered osmium tetroxide fixation, Epon embedding, and uranyl acetate staining. The basic techniques currently used for isolating nuclei and nuclear components from this cell for light microscopy have been described in detail by Gall (1), Callan and Lloyd (2), and, for the specific study of nucleoli, by Macgregor (3). The techniques used to prepare isolated nucleoli for electron microscopy are similar to those described for handling lampbrush chromosomes (4).

Briefly, the isolation procedure (using dissecting microscope, jewelers' forceps, and pipette) consists of isolation and cleaning of the oocyte nucleus followed by isolation of the nuclear contents in a well slide or other chamber by manual removal of the nuclear envelope. The effects of various chemicals, pH, medium tonicity, and enzymes on the nuclear components can be observed by phase contrast microscopy either by including the agent in the original medium or by adding the agent or changing the medium while observing the isolated components. Preparation of similar material for electron microscopy consists of isolating and treating on a grid with supporting film previously placed in the bottom of the chamber. In this case, the material is centrifuged, precipitated on the grid, stained with uranyl acetate, and dried before observation with the electron microscope.

OBSERVATIONS

At pachytene the *Triturus* oocyte nucleus contains one or two nucleoli attached to chromosomes. As the oocyte enlarges into the lampbrush chromosome stage (an extended diplotene), scores of nucleoli (ranging from 0.5–10 μ in diameter) appear close to the nuclear envelope (figs. 1

and 3). By the time the oocyte is 400–500 μ in diameter (about one fourth its mature diameter), approximately the maximum number of peripheral nucleoli already are present in the nucleus. These nucleoli slowly increase in size and remain close to the envelope until the oocyte is 1200–1300 μ in diameter. At this stage each nucleolus fragments into a cluster of interconnected smaller spheres and migrates toward the center of the nucleus, where the nucleoli form a tight layer around the chromosomes which are now contracting out of the lampbrush condition toward diakinesis.

Fine Structure In Situ

In thin sections, the pachytene nucleoli typically show a coarsely fibrous core surrounded by a granular cortex. Until the oocyte reaches 300–400 μ in diameter, the small peripheral nucleoli are structurally similar to the core of the pachytene nucleoli but show no granular cortex (fig. 3). Although in sections these nucleoli clearly are separated from and show no apparent connection with the nuclear envelope, they are firmly attached to the envelope when the nuclear contents are isolated (fig. 1). When the oocyte reaches 400–500 μ in diameter, a granular cortex component appears on each nucleolus as a thin shell (fig. 2), which continues to enlarge until the bipartite morphology of the typical nucleolus of medium size oocytes is reached (fig. 5). When this granular element appears, the nucleoli do not remain attached to the envelope and fall free during isolation of the nuclear contents. When the nucleoli fragment into clusters in the larger oocytes, each smaller sphere retains the bipartite morphology of the parent nucleolus. Staining and enzymatic digestion show that both the core and cortex components contain RNA and protein.

Intranucleolar DNA

When nucleoli of either medium-sized oocytes (typical bipartite morphology) or maturing oocytes (cluster stage) are isolated into 0.05–0.15 M potassium or sodium chloride solutions, the core and cortex components remain together. When the molarity is lowered to 0.025 M or less, the granular component separates from the cores and disperses in the isolation medium, the speed of separation and dispersal increasing with decreasing molarity. Under these conditions, the cores of the spherical nucleoli from medium-sized oocytes expand into loose fibrous networks whereas those from the clusters appear as circles resembling loosely beaded necklaces of varying sizes (figs. 4 and 6). The size of each individual network or circle is directly correlated with the size of the nucleolus from which it was derived. In the later stage, the sizes of cores from clusters range from single small beads to large necklaces up to 200 μ in circumference. Measurements of necklaces from single nuclei suggest that the sizes of the circles form a geometrical progression. Measurements from one nucleus, for example, showed classes of circles approximately 11.25, 22.5, 45, 90, and 180 μ in circumference (smaller circles were not measured).

Treatment of the nucleolar cores with trypsin or ribonuclease decreases their size, but no breaks occur in the continuity of fibers or circles (figs. 7 and 8). Deoxyribonuclease digestion results in a rapid fragmentation and dispersal of the fibrous networks and interbead breakage of the necklaces and decrease in bead size until most of the beads show independent movement (fig. 9). Electron microscopy of isolated cores shows that the continuity of the fibrous networks and the interbead strands of the necklaces is maintained by a ~ 30 Å axial fiber unevenly coated with a matrix material (figs. 10 and 11). This axial fiber is similar in diameter to the DNA-containing axes of the lateral loops of the lampbrush chromosomes from the same cell (4). The decrease in circle sizes with trypsin or ribonuclease digestion presumably does not involve removal of DNA, but rather a coiling of the DNA similar to that occurring in lateral loop axes following such treatment (5).

Intranucleolar "Membrane"

When nuclei from medium-sized oocytes are isolated in 0.1 M phosphate buffer (pH 7) previous to isolating the nuclear contents into low-molarity saline, the speed at which the nucleolar core and cortex components separate is reduced. Under these conditions, a low-contrast spherical object, usually 1–4 μ in diameter, is found attached to many of the expanded fibrous cores. The absence of this component from cores isolated without use of phosphate buffer is due to the fact that it is always detached during the more rapid separation of core and cortex which occurs when only potassium or sodium chloride solutions are used in the isolation procedure. Under the latter condition, the detached structures rapidly migrate from the area of the lampbrush chromosomes as spherical vesicles on the front edge of the circle of dispersing nucleoplasm. A small basal portion of this component, however, remains with each core and appears as a small dark dot or thickened region on the core component.

The effect of the low-molarity saline in detaching the low-contrast component can be reduced further by adding 0.1–0.2 M sucrose to the final isolation medium. In this medium most of the cores retain the low-contrast object, but its shape now varies from a spherical or bladder-like structure on a narrow stalk to a thin filament up to 25 μ in length (fig. 12). If preparations are observed immediately after isolation, when the nucleoli are still in their original orientation, this component can be seen to be attached to each core at the point which was closest to the nuclear envelope.

When spherical or bladder-like in shape, this structure contracts markedly when prepared for electron microscopy and is opaque in the electron beam (fig. 13). When the long filaments are observed with the electron microscope they appear as flattened tubules 500 Å to 0.5 μ in diameter which usually contain irregularly spaced granules 300 to 1000 Å in diameter (fig. 14).

Although no direct evidence is available, this component appears to be membranous in nature. Both its phase contrast and electron transmission

characteristics are similar to those of known membranous structures when these are treated in the same manner, *e.g.*, the nuclear envelope, bacterial ghosts, and vesicles from membrane fractions of density gradient preparations of ribosomes. Also, treatment of this component with acidic solutions (pH 2) or with detergents, such as sodium dodecyl sulfate, gives results similar to those obtained with the nuclear envelope, *i.e.*, both structures immediately break down and disappear in the medium.

DISCUSSION

The presence (positive Feulgen reaction) of extrachromosomal DNA in peripheral nucleoli of amphibian oocytes has been reported by several authors (6-8). Indirect evidence for DNA in peripheral nucleoli of *Triturus* oocytes has been reported (9), based on actinomycin D sensitivity of nucleolar RNA synthesis and *in vitro* synthesis of nucleolar RNA. More recently, two authors independently reported DNA in peripheral nucleoli of *Triturus* (10) and *Plethodon* (11) oocytes and showed that this DNA is present, at least during part of oogenesis, in circles of various sizes.

Labeling with radioactive RNA precursors shows that rapid synthesis of RNA occurs in *Triturus* oocyte nucleoli (9, 12). Base composition analyses have shown that the nucleolar RNA of *Triturus* oocytes is similar in composition to the bulk cytoplasmic RNA, which is primarily ribosomal (13). In addition, sedimentation values and labeling patterns of nucleolar RNA isolated from *Triturus* oocytes indicate that ribosomal RNA precursor molecules (40S) are present in these nucleoli (14).

These facts suggest that the large number of extrachromosomal DNA-containing nucleoli in *Triturus* oocytes (and most probably in oocytes of other amphibians) results from an amplification of the ribosomal RNA cistrons without concurrent duplication of the rest of the genome in the same cell. This amplification apparently results in the accumulation and storage of ribosomes in the oocyte for use during the early developmental stages of the embryo (15).

The mechanisms by which this amplification occurs and the free nucleoli are spaced next to the nuclear envelope remain obscure. There is strong evidence that the ribosomal RNA cistrons normally are contained in the nucleolar organizer loci which occur at constant sites on specific chromosomes (16, 17). Judged from the number of nucleoli present, the pachytene-stage cells, which give rise to the larger oocytes with peripheral nucleoli, contain the normal number of nucleolar organizers. The obvious inference is that the ribosomal RNA cistrons must, in some manner, be detached from the chromosomes without impairing the genome for future cell generations. Two possible alternatives (not mutually exclusive) are obvious: 1) nucleolar organizer loci on the chromosomes are repetitively duplicated and the copies detached, followed by dispersal of the copies over the inner surface of the nuclear envelope; or 2) a single copy (or limited number of copies) is made and detached. This copy moves to the

nuclear envelope where a sequence of further duplications occurs to produce the final number of peripheral nucleoli. Both alternatives have been considered for the ring nucleoli in *Plethodontid* oocytes (11). Close proximity of equal-sized nucleoli next to the nuclear envelope is sometimes observed in *Triturus* (fig. 1), which suggests that division of nucleoli may be occurring there. Without labeling data, however, definitive support for either of these possibilities is lacking so far. In both cases, the wide range in the length of the DNA contained in the core component of these nucleoli would introduce a further complication to any mechanism of amplification. The DNA necklaces appear to fall into a 1-2-4-8 . . . progression in circumference sizes, suggesting that a doubling of the nucleolar DNA component is occurring. It has been suggested (18) that this could occur by duplication of a ring followed by breakage and fusion of adjacent ends to form a ring of exactly twice the circumference of the original one.

Although the presence of lipoproteins in nucleoli has been reported previously [see (19) for references], this is the first report of a membranous component within nucleoli. The function of this structure is unknown. However, the first incorporation of radioactive RNA precursors occurs inside these nucleoli at a point eccentric toward the nuclear envelope (20). This is also the point where the membranous component is attached to the DNA-containing core of the nucleolus. The coincidence of attachment point and site of initial RNA synthesis suggests that the membranous nucleolar component may play a role in the intranucleolar transformation and/or transfer of the large ribosomal RNA precursor molecules following their transcription on the DNA in the nucleolar core.

RESUMEN

Los núcleos de los ovocitos de *Triturus* de tamaño mediano contienen varios cientos de nucleolos localizados periféricamente no unidos a los cromosomas. En cortes finos, cada nucleolo exhibe un núcleo fibroso rodeado de una corteza granular. En ovocitos mayores, estos nucleolos se fragmentan en agrupamientos de esferas más pequeñas, reteniendo cada una de ellas la morfología bipartita del nucleolo progenitor. Cuando se aíslan los nucleolos de cada estadio en solución salina molar de menos de 0,025, el componente granular se dispersa rápidamente. En estas condiciones, los núcleos fibrosos nucleolares de los ovocitos de tamaño mediano se expanden en redes fibrosas laxas, mientras que los de los agrupamientos nucleolares aparecen como círculos que semejan collares de cuentas ensartadas en forma laxa, de diversos tamaños. El tratamiento de los núcleos con tripsina o RNasa disminuye su tamaño, pero no produce rupturas en la continuidad de las fibras o círculos. La digestión con DNasa da por resultado la desintegración de los núcleos. La microscopía electrónica de núcleos aislados muestra que la continuidad de las redes fibrosas y los filamentos existentes entre las cuentas de los collares es mantenida por una fibra de 30 Å de diámetro. Cuando el componente nucleolar granular se dispersa por métodos más moderados, puede observarse una estructura de bajo contraste de fase unida al lado de cada núcleo fibroso más cercano a la envoltura nuclear. Dependiendo del estadio del ovocito o del medio de aislamiento empleado, este componente puede mantener una forma esférica o aparecer como un fino filamento de hasta 24μ de longitud.

Con microscopía electrónica, estos filamentos aparecen como túbulos membranosos aplastados que varían de ancho desde 500Å a 0.5μ y con frecuencia contienen gránulos espaciados irregularmente de hasta 1000Å de diámetro. La evidencia de otros estudios muestra que la primera incorporación de precursores ARN ocurre en un sitio del nucleolo que coincide con el punto de unión de este componente membranoso y que la incorporación probablemente sea en las moléculas precursoras del ARN ribosómico. Se sugiere que el componente nucleolar membranoso puede desempeñar un papel en la transferencia o transformación intranucleolar de las moléculas precursoras del ARN ribosómico después de su transcripción en el ADN de los núcleos fibrosos nucleolares.

REFERENCES

- (1) GALL, J. G.: Lampbrush chromosomes from oocyte nuclei of the newt. *J Morph* 94: 283-352, 1954.
- (2) CALLAN, H. G., and LLOYD, L.: Lampbrush chromosomes of crested newts *Triturus cristatus* (Laurenti). *Phil Trans Roy Soc London*, ser B 243: 135-219, 1960.
- (3) MACGREGOR, H. C.: The role of lampbrush chromosomes in the formation of nucleoli in amphibian oocytes. *Quart J Micr Sci* 106: 215-228, 1965.
- (4) MILLER, O. L., JR.: Fine structure of lampbrush chromosomes. *Nat Cancer Inst Monogr* 18: 79-99, 1965.
- (5) CALLAN, H. G.: The nature of lampbrush chromosomes. *Int Rev Cytol* 15: 1-34, 1963.
- (6) BRACHET, J.: La localisation de l'acide thymonucléique pendant l'ovogenèse et la maturation chez les Amphibiens. *Arch Biol (Liege)* 51: 151-165, 1940.
- (7) PAINTER, T. S., and TAYLOR, A. N.: Nucleic acid storage in the toad's egg. *Proc Nat Acad Sci USA* 28: 311-317, 1942.
- (8) GUYÉNOT, E., and DANON, M.: Chromosome et ovocytes des Batraciens. *Rev Suisse Zool* 60: 1-129, 1953.
- (9) IZAWA, M., ALLFREY, V., and MIRSKY, A.: Composition of the nucleus and chromosomes in the lampbrush stage of the newt oocyte. *Proc Nat Acad Sci USA* 50: 811-817, 1963.
- (10) MILLER, O. L., JR.: Extrachromosomal nucleolar DNA in amphibian oocytes. *J Cell Biol* 23: 60A, 1964.
- (11) PEACOCK, W. J.: Chromosome replication. *Nat Cancer Inst Monogr* 18: 101-131, 1965.
- (12) GALL, J. G.: Uptake of nucleic acid precursors by oocytes of the newt (*Triturus viridescens*). *Genetics* 44: 512, 1959.
- (13) EDBSTRÖM, J.-E., and GALL, J. G.: The base composition of ribonucleic acids in lampbrush chromosomes, nucleoli, nuclear sap, and cytoplasm of *Triturus* oocytes. *J Cell Biol* 19: 279-284, 1963.
- (14) GALL, J. G.: Nuclear RNA of the salamander oocyte. *Nat Cancer Inst Monogr* 23: 475-478, 1966.
- (15) BROWN, D. D.: The nucleolus and synthesis of ribosomal RNA during oogenesis and embryogenesis. *Nat Cancer Inst Monogr* 23: 297-309, 1966.
- (16) BIRNSTIEL, M. L., WALLACE, H., SIRLIN, J. L., and FISCHBERG, M.: Localization of the ribosomal DNA complements in the nucleolar organizer region of *Xenopus laevis*. *Nat Cancer Inst Monogr* 23: 431-447, 1966.
- (17) RITOSSA, F. M., ATWOOD, K. C., LINDSLEY, D. L., and SPIEGELMAN, S.: On the chromosomal distribution of DNA complementary to ribosomal and soluble RNA. *Nat Cancer Inst Monogr* 23: 449-472, 1966.
- (18) MCCLINTOCK, B.: Personal communication, 1964.
- (19) BUSCH, H., BYVOET, P., and SMETANA, K.: The nucleolus of the cancer cell: a review. *Cancer Res* 23: 313-339, 1963.
- (20) GALL, J. G.: Personal communication, 1965.

PLATE 8

FIGURE 1.—Portion of isolated nuclear envelope from young oocyte with peripheral nucleoli attached. Pairs of approximately equal-sized nucleoli are indicated. Phase contrast. $\times 1,300$

FIGURE 2.—Thin section of nucleolar margin at stage of oogenesis (oocytes about one fourth maximum diameter) when the granular cortex component appears on the peripheral nucleoli. $\times 16,500$

FIGURE 3.—Thin section of peripheral nucleoli during early stage of oogenesis when the nucleoli lack a granular cortex. $\times 4,000$

FIGURE 4.—Isolated nucleolar cores after removal of granular cortex component in low-molarity saline. Note range (*arrows*) in size of cores from different nucleoli. Phase contrast. $\times 650$

FIGURE 5.—Thin section showing typical bipartite morphology of peripheral nucleoli in medium-sized oocytes. $\times 17,000$

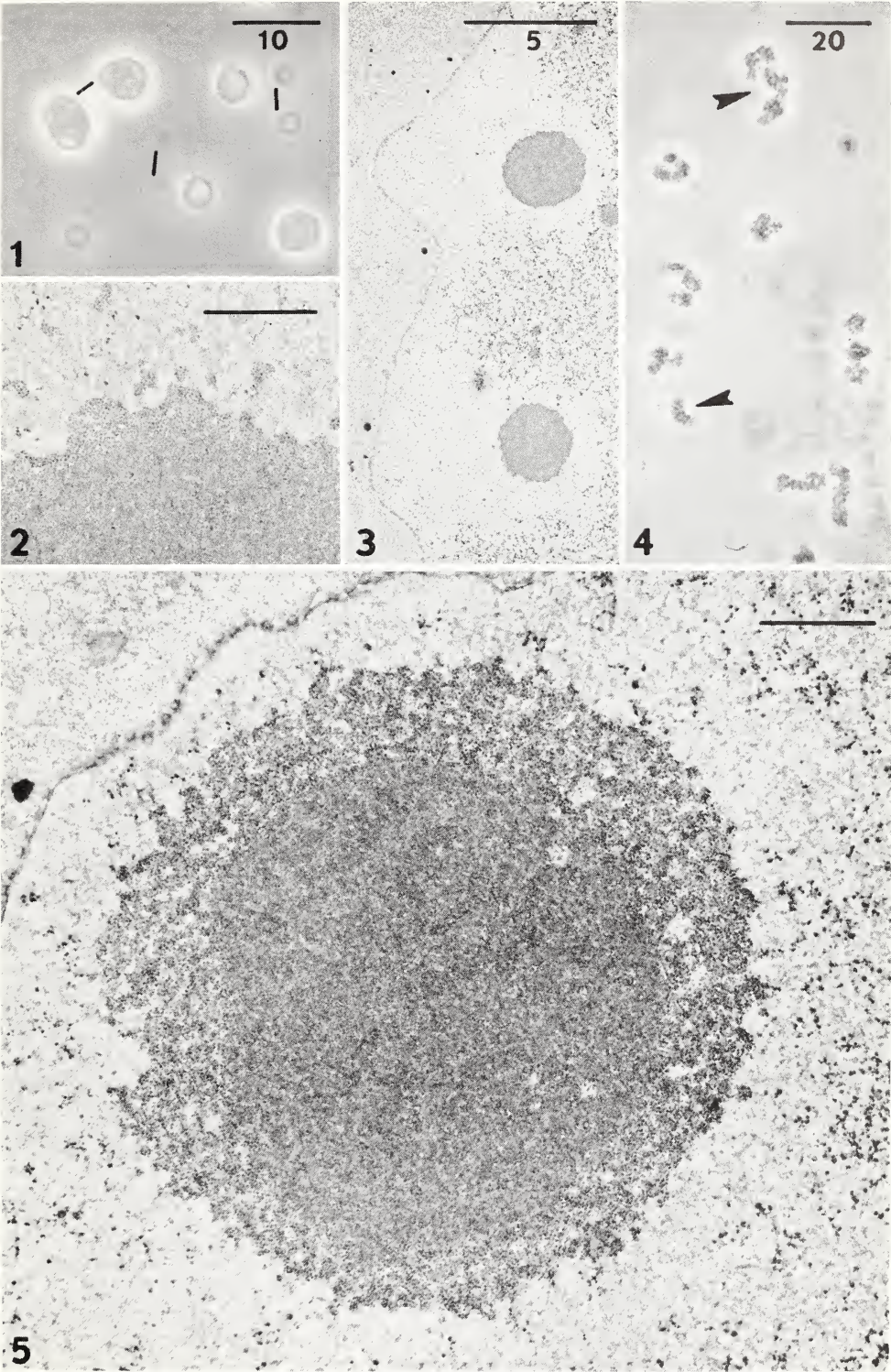


PLATE 9

FIGURE 6.—Nucleolar cores in circular form derived from cluster nucleoli during late stages of oogenesis by isolation in low-molarity saline. Largest circles are approximately $165\ \mu$ in circumference. Linear cores presumably result from opening of the circles by mechanical breakage during isolation. Phase contrast. $\times 240$

FIGURE 7.—Sequential stages in the digestion of circular nucleolar cores isolated into a medium containing trypsin. Phase contrast. $\times 255$

FIGURE 8.—Same as figure 7 except cores isolated into medium containing ribonuclease. $\times 255$

FIGURE 9.—Same as figure 7 except cores isolated into medium containing deoxyribonuclease. $\times 255$

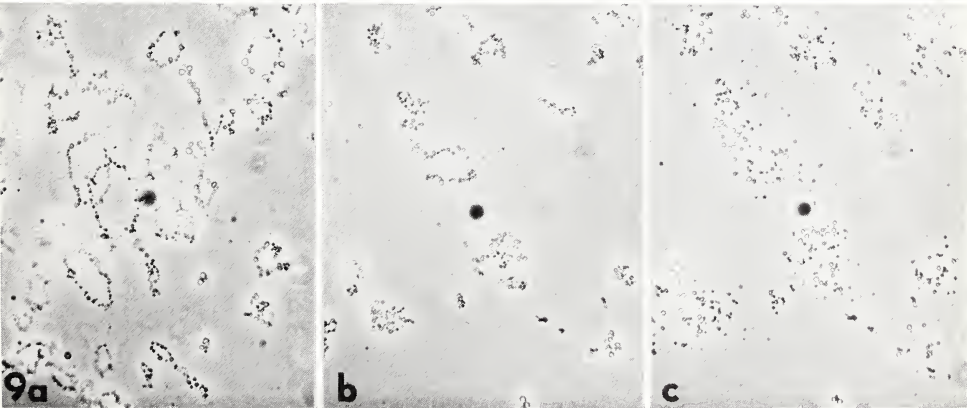
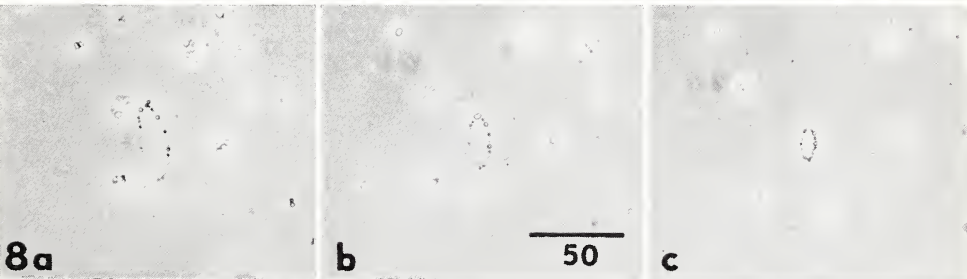
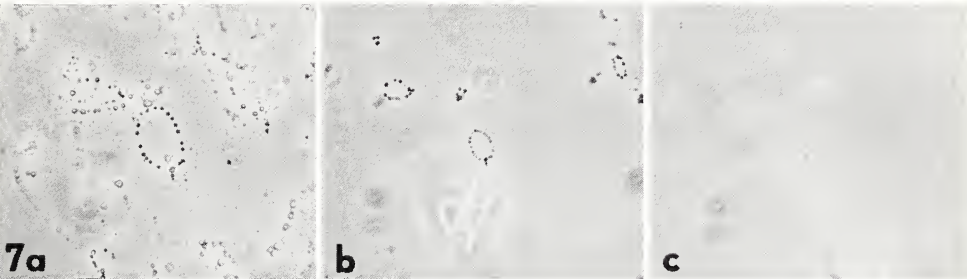
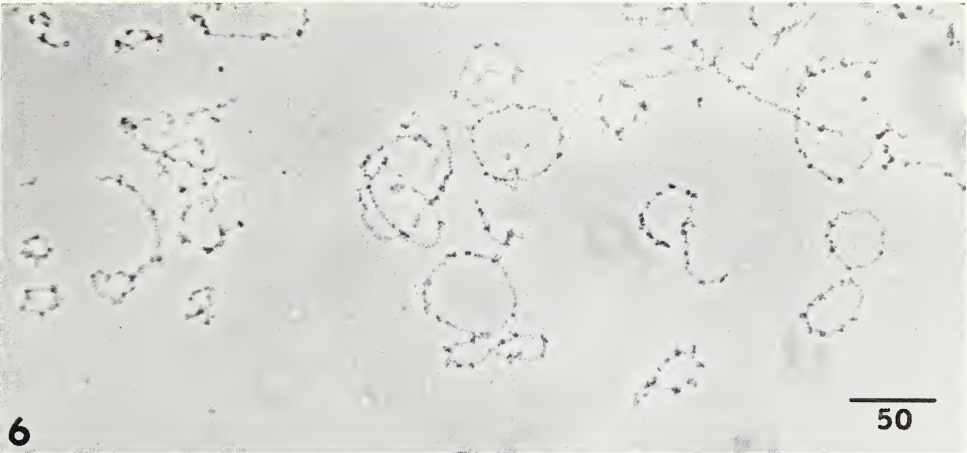


PLATE 10

FIGURE 10.—Electron micrograph of circular nucleolar core isolated in low-molarity saline without previous treatment with phosphate buffer. A portion of the core was broken away in the preparation for microscopy. $\times 3,400$

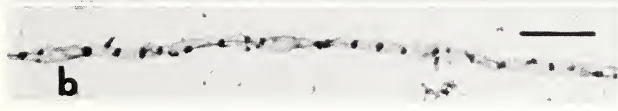
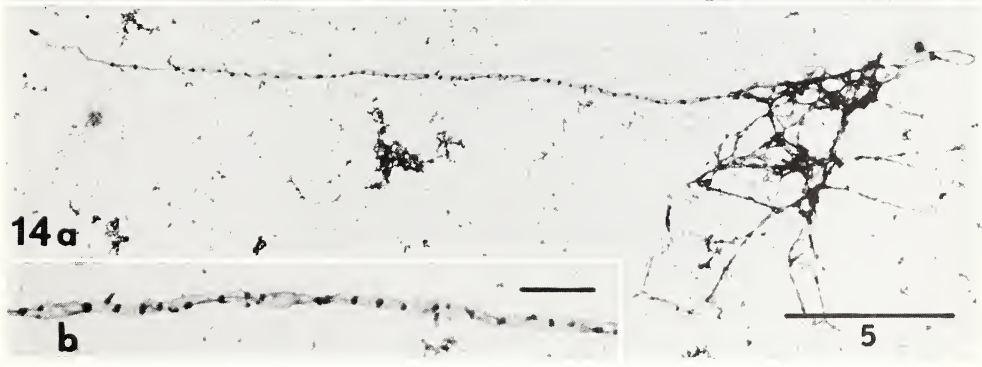
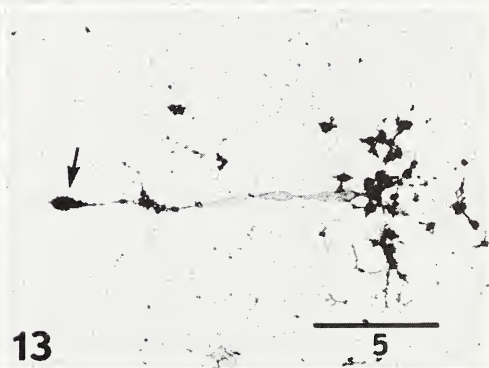
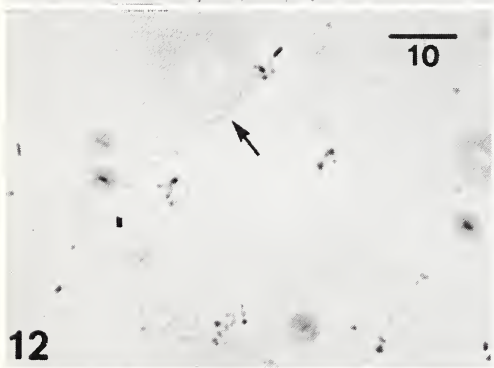
FIGURE 11.—Electron micrograph of nucleolar core isolated from medium-sized oocyte. The nucleus was isolated in phosphate buffer previous to removal of its contents in low-molarity saline. A membranous component (*arrow*) is attached to the core. $\times 2,700$

FIGURE 12.—Nucleolar cores from medium-sized oocyte with attached membranous tubules (*arrow*). The nucleus was isolated in phosphate buffer followed by isolation of the nuclear contents into low-molarity saline with sucrose. Phase contrast. $\times 900$

FIGURE 13.—Preparation similar to figure 11. Vesicle (*arrow*) at end of membranous component collapsed during precipitation for electron microscopy and is opaque to the electron beam. $\times 3,400$

FIGURE 14a.—Electron micrograph of preparation similar to figure 12. The membranous tubule contains granules irregularly spaced along its length. $\times 4,500$

FIGURE 14b.—Higher magnification of a portion of the tubule shown in figure 14a. $\times 9,800$



DISCUSSION

Sirlin: Have you made a control by separating the lampbrush chromosomes from the nucleoli and then seeing if you get the DNA rings?

Miller: The chromosomes can be removed by isolating the nuclear contents on top of a fine-meshed electron microscope grid, thereby collecting the chromosomes on the grid and allowing the nucleoli to fall to the bottom of a well-slide or microcentrifuge tube. The rings are still present, and the role of DNA in maintaining the continuity of the rings can still be demonstrated with deoxyribonuclease.

Sirlin: Is the membranous filament coming out of the core deoxyribonuclease sensitive?

Miller: The membranous filament does not break down with deoxyribonuclease. However, the cores are completely disintegrated by deoxyribonuclease. This allows the membranous components to float free in solution, apparently unaffected by the enzyme.

Pavan: I would like to ask if all of these nucleoli come from the nucleolar organizer region, or do you think there are other sites in the nucleus where nucleoli are produced?

Miller: I cannot answer the question. There is evidence that the nucleolar organizer locus is present on a specific chromosome at the stage when lampbrush chromosomes usually are isolated, *i.e.*, when a large number of peripheral nucleoli already are present. The nucleolus at this locus may be small, medium, or large, depending on the preparation. By the time the oocyte is 350 to 400 μ in diameter, approximately the total number of nucleoli are present. It is difficult to examine isolated components of the nucleus before this stage. At present, I have no evidence as to whether the peripheral nucleoli are formed at this chromosomal site repetitively, or are formed by an organizer replicating at an extrachromosomal location. Either way, it occurs relatively early in the growth of these oocytes.

Busch: You have suggested that the 45S RNA of the nucleolus is the precursor of the 28S and 18S RNA. The evidence that it is the precursor of 28S RNA is very sound. The evidence that it is the precursor of 18S RNA is very dubious. The second point relates to the "granules" which Dr. Bernhard discussed. One is not certain that these structures are granules, but rather they are granular elements, which could very well be just kind of twisted ends of thick fibrillar elements.

Miller: It is possible with this system to isolate the nucleoli into a salt solution in which the nucleolar core and cortex components stay together, then to make the solution hypotonic by introducing distilled H_2O . In the hypotonic solution the granular component disperses. If the preparation is precipitated at the beginning of this dispersal, one can demonstrate that there are granules plus a fibrous component in the cortex.

Birstiel: How much RNA can the DNA contained in the nucleolar cores code for in terms of 45S units or ribosomal RNA subunits?

Miller: I cannot answer your question, but can give you an idea of the apparent length of the DNA. In the species used in this study the nucleolar rings range up to 200 μ in circumference and we know that there is DNA in the beads on the necklaces also. Five hundred rings averaging 25 μ in circumference per oocyte would be a conservative estimate of the length of DNA in the peripheral nucleoli. How much of this actually is used for transcription is anyone's guess.

Barr: Do you think that the DNA in these nucleoli is the same material that was described in the mid-40's by Painter and Taylor [ref. (7) this paper] in the micro-nucleoli of toad oocytes?

Miller: I believe it is the same.

Ultrastructure and Mode of Formation of the Nucleolus in Plant Cells^{1,2}

J. G. LAFONTAINE and Fr. A. LORD, *Biology Department, Faculty of Sciences, Laval University, Quebec, Canada*

SUMMARY

The ultrastructure of the nucleolus is rather constant in a wide variety of plant species. This organelle exhibits two main types of zones each predominantly consisting of ribosome-like granules or fine fibrils. Dense particles, 200 Å or slightly more in diameter, are often associated with the fibrillar zones. The distribution and relative size of these two types of zones vary with species as well as from tissue to tissue. It is also generally observed that chromatin is intimately associated with the nucleolar mass. The early and midtelophase nuclei, in most species examined, are characterized by large patches of Feulgen-negative material which is assumed to condense into nucleolar bodies. The fact that this sub-

stance is just as abundant in species with small chromosomes argues strongly against the hypothesis that it originates from a chromosomal matrix. In plants where nucleoli often persist till late telophase, the process of nucleolar formation is similar to that normally observed in other species: A thin coating is first observed on the surface of the early telophase chromosomes and abundant pre-nucleolar material subsequently appears within the nucleus. Since the bulk of the new nucleolus is not derived from pre-existing nucleolar material, it seems reasonable to postulate that it is synthesized within the telophase nucleus.—*Nat Cancer Inst Monogr* 23: 67–75, 1966.

ORGANIZATION OF PLANT NUCLEOLI

IT HAS LONG been realized from light microscopical observations that plant nucleoli are quite heterogeneous in structure. Some earlier workers assumed that this composite organization of nucleoli was due to the presence of vacuoles of various sizes, but others believed that such heterogeneity reflected a dual chemical makeup of these organelles (1, 2).

More recent studies have shown that both of these factors do in fact contribute to the heterogeneous appearance of plant nucleoli. Under electron microscopy, nucleoli indeed often exhibit large vacuoles, usually

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This work was supported by grant T-1018 from the National Research Council of Canada.

centrally located, as well as numerous smaller ones. Zones of different staining intensity and ultrastructure are also found within their mass. In all cases described so far, such zones have been shown to consist predominantly either of ribosome-like particles or fibrils 60–100 Å in diameter (3–7). These zones are readily visualized in 1 μ sections stained with methylene blue (fig. 1).

Consecutive thick (1 μ) and thin sections examined under light and electron microscopy, respectively, reveal that the more intensely stained nucleolar regions correspond to the fibrillar zones, whereas the remaining lighter portions consist mostly of granular material. Although the distribution and relative size of these zones vary in different species, they represent a constant feature of meristematic cell nucleoli in a wide variety of plants. Serial sections permit the demonstration, moreover, that zones of a given type located in various portions of the nucleolus are in fact often continuous with one another and thus form coarse three-dimensional patterns extending throughout its mass.

Differences in the degree of packing of the particles and fibrils are assumed (6) to account for part of the distinctive staining intensity of these areas. It is also conceivable that the presence of a more amorphous substance within the fibrillar zones (7) contributes to their greater density and staining intensity as compared to the granular areas. The observation that these two types of nucleolar zones still differ in density following digestion with ribonuclease (8) indeed suggests that they contain other substances, possibly proteins, in different amounts.

Besides the zones just referred to, plant nucleoli also exhibit Feulgen-positive portions (fig. 8) which, under electron microscopy, are characterized by a distinct texture (9). Since Heitz's (10) original studies, evidence has accumulated showing that specific segments of certain chromosomes are intimately associated with the nucleolus and may actually go through its mass. Such intranucleolar chromatin strands have been identified under electron microscopy in a number of species (3, 11, 12) and appear to be a common feature of nucleoli in plants with either reticulate or prochromosomal nuclei (6). In a given ultrathin section, the intranucleolar chromatin zones do not, naturally, all exhibit continuity with chromosomal strands located outside this organelle; for this reason, it is often difficult to ascertain whether some of these areas correspond to nucleolar organizing segments of chromosomes. Examination of serial 0.5 μ sections shows, however, that a large proportion of the Feulgen-positive nucleolar portions are indeed part of nucleolar chromosomes and should not, therefore, be visualized as typical inclusions. Further work is being undertaken to estimate the importance of these chromatin zones relative to the total nucleolar volume and verify to what extent they might contribute to the high DNA content recently revealed in plant nucleoli by biochemical analysis (13).

MODE OF FORMATION OF THE NUCLEOLUS AT TELOPHASE

The problem of the re-formation of the nucleolus at telophase has been the object of numerous studies in the past. Such investigations have furnished much data concerning the condensation of these organelles onto specific chromosomal sites (10, 14), as well as their number, form, and size in different plant species. However, opinions are still divided as to their origin and the role of the nucleolar chromosomes during their formation at telophase.

The main problem at issue, regarding the origin of the nucleolar material, is whether it is derived from a chromosomal matrix (14-16), from the spindle (17, 18), or simply synthesized anew within the telophase nucleolus (5, 19). As is now well established, bodies of various sizes and shapes, or a diffused Feulgen-negative material (figs. 3-5), are observed within the re-forming nucleus prior to the appearance of a nucleolus. The observation that the substance in question gradually decreases in amounts during growth of the nucleolus has generally been interpreted as evidence that it is prenucleolar in nature (2, 5, 14, 16-18, 20, 21).

To examine this hypothesis further, some 20 plant species with reticulate or prochromosomal (16, 20, 22) interphase nuclei were studied by means of correlated light and electron microscopic techniques. In most plants investigated (6, 9), the early telophase chromosomes were characterized by a thin coating of material which by midtelophase completely enrobes the chromosomes (figs. 3-5), as already described for *Vicia faba* meristematic cells (5). This material stains metachromatically with azure B and loses this characteristic, as does the nucleolus, following digestion with ribonuclease (figs. 5 and 6). Arguments favoring the view that this material condenses to form the nucleolar bodies are presented elsewhere (6).

It is interesting that, in prochromosomal species with small chromosomes and large nucleoli (figs. 4 and 7), this substance is just as abundant as in other plants. To all appearances, its volume by midtelophase equals or may even exceed that of the chromosome set. A similar situation prevails in several other plant species, according to Doutreligne (16). One may, therefore, safely conclude that the bulk of this prenucleolar material cannot possibly correspond to a matrix extruded from the telophase chromosomes.

A smaller proportion of the plants examined showed numerous RNA-containing bodies (8) which, in accord with recent electron microscopic studies (21, 23) of animal material, are assumed to coalesce into nucleoli.

Our observations have furnished additional data supporting the view that the bulk of the Feulgen-negative material referred to above is not derived from pre-existing elements of nucleolar origin. In *Helianthus annuus* (9), it is indeed noted that the nucleolus which normally disperses at late prophase may also occasionally give rise to diffuse masses that accompany the chromosome sets to the poles or may persist in the form of

large bodies. The fact that such bodies remain intact until later stages of telophase and coexist with new nucleoli arising within the nucleus (fig. 2), as also noted in other plant species (6), argues against the suggestion (24) that preformed nucleolar material gives rise to this organelle.

RESUMEN

Se ha encontrado que la ultraestructura del nucleolo es más bien constante en una gran variedad de especies de plantas. Este organelo exhibe dos tipos principales de zonas, cada una de las cuales consiste predominantemente de gránulos semejantes a ribosomas o finas fibrillas. Partículas densas, de 200 Å o poco más de diámetro, se asocian con frecuencia a las zonas fibrilares. La distribución y el tamaño relativo de estos dos tipos de zonas varían con las especies así como de tejido a tejido. También por lo general se observa que la cromatina está íntimamente asociada con la masa nucleolar. Los núcleos telofásicos tempranos y medios, en la mayoría de las especies examinadas, se caracterizan por grandes bloques de material Feulgen negativo que presumiblemente se condensan en cuerpos nucleolares. El hecho de que esta sustancia sea tan abundante en especies con cromosomas pequeñas arguye en forma poderosa contra la hipótesis de que se origina de una matriz cromosómica. En plantas en que los nucleolos a menudo persisten hasta la telofase tardía, el proceso de formación nucleolar es similar al que normalmente se observa en otras especies: primero se observa un fino recubrimiento en la superficie de los cromosomas telofásicos tempranos y aparece posteriormente abundante material prenucleolar dentro del núcleo. Puesto que la mayor parte del nuevo nucleolo no deriva del material nucleolar preexistente, parece razonable postular que se sintetiza dentro del núcleo telofásico.

REFERENCES

- (1) ZIRKLE, C.: Nucleoli of the root tip and cambium of *Pinus strobus*. *Cytologia* 2: 85-104, 1931.
- (2) GATES, R. R.: Nucleoli and related nuclear structures. *Bot Rev* 8: 337-409, 1942.
- (3) PORTER, K. R.: Problems in the study of nuclear fine structure. In *Proceedings of the Fourth International Congress of Electron Microscopy*, Berlin, 1958 (Bargmann, W., et al., eds.). Berlin, Springer-Verlag, 1960, vol 2, pp 186-199.
- (4) FABBRI, F.: Ricerche al microscopio ottico ed elettronico sulla struttura del nucleolo di cellule meristematiche di alcune piante e considerazioni critiche sulla teoria del "Nucleolonema." *Caryologia* 16: 715-749, 1963.
- (5) LAFONTAINE, J. G., and CHOUINARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167-201, 1963.
- (6) LAFONTAINE, J. G.: Structural components of the nucleus in mitotic plant cells: A review. In *Ultrastructure in Biological Systems. The Nucleus* (Dalton, A. J., and Hagenau, F., eds.). New York and London, Academic Press Inc., vol 2. In press.
- (7) HYDE, B. B., SANKARANARAYANAN, K., and BIRNSTIEL, M. L.: Observations on fine structure in pea nucleoli in situ and isolated. *J Ultrastruct Res* 12: 652-667, 1965.
- (8) LORD, Fr. A.: Unpublished data.
- (9) LAFONTAINE, J. G.: Manuscript in preparation.

- (10) HEITZ, E.: Nukleolen und Chromosomen in der Gattung *Vicia*. *Planta Med* 15: 495-505, 1931.
- (11) LAFONTAINE, J. G.: Structure and mode of formation of the nucleolus in meristematic cells of *Vicia faba* and *Allium cepa*. *Biophys Biochem Cytol* 4: 777-784, 1958.
- (12) POUSSEL, H., and GAVAUDAN, P.: Etude au microscope électronique des relations entre le nucléole et la chromatine chromosomique dans les noyaux interphasiques de *Hyacinthus orientalis*. *C R Acad Sci (Paris)* 253: 2398-2400, 1961.
- (13) MCLEISH, J.: Deoxyribonucleic acid in plant nucleoli. *Nature (London)* 204: 36-39, 1964.
- (14) MCCLINTOCK, B.: The relationship of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
- (15) MARSHAK, A. G.: The morphology of the chromosomes in *Pisum sativum*. *Cytologia* 2: 318-339, 1931.
- (16) DOUTRELIGNE, J.: Chromosomes et nucléole dans les noyaux du type euchromocentrique. *Cellule* 42: 31-101, 1933.
- (17) RATTENBURY, J. A., and SERRA, J. A.: Types of nucleolus reconstitution in telophase and the question of the "Nucleolar organizer." *Port Acta Biol Ser A3*: 239-260, 1952.
- (18) YASUZUMI, G., and SUGIHARA, R.: The fine structure of nuclei as revealed by electron microscopy. II. The process of nucleolus reconstitution in Ehrlich ascites tumor cell nuclei. *Exp Cell Res* 40: 45-55, 1965.
- (19) SWIFT, H.: Studies on nucleolar function. In *A Symposium on Molecular Biology* (Zirkle, R. E., ed.). Chicago, Univ Chicago Press, 1958, pp 266-303.
- (20) MANTON, I.: Some new evidence on the physical nature of plant nuclei from intra-specific polyploids. *Proc Roy Soc [Biol]* 118: 522-547, 1935.
- (21) STEVENS, B. J.: The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J Cell Biol* 24: 349-368, 1965.
- (22) DANGEARD, P.: *Cytologie végétale et cytologie Générale*. Paris, P. Lechevallier, 1947, 611 pp.
- (23) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. I. The nucleus of *Bradysia mycorum* Frey (Sciaridae), with special reference to the nucleolus. *J Cell Biol* 17: 153-165, 1963.
- (24) BROWN, S. W.: Mitosis and meiosis in *Luzula campestris*. *Univ Calif Publ Bot* 27: 231-278, 1954.

PLATE 11

FIGURE 1.—*Raphanus sativus* interphase nucleus. The nucleolus (n) in this species is quite large and characterized, moreover, by easily detectable zones of different density. The denser areas extend from the surface of the nucleolus to its more centrally located regions. $\times 7,500$

FIGURE 2.—Midtelophase nucleus from *Helianthus annuus* meristematic cell. Besides the nucleolus (nl) in the process of formation within the nucleus, a large persisting nucleolar mass (arrow) may also be seen at the cell pole. The section $1\ \mu$ thick was stained by the Feulgen procedure and methylene blue. $\times 5,500$

FIGURE 3.—Light micrograph of midtelophase nucleus from *Lathyrus odoratus* meristematic cell. Large amounts of prenucleolar material fill the spaces (arrows) between the chromosomes. Stained by the Feulgen procedure and methylene blue. $\times 5,500$

FIGURE 4.—Midtelophase nucleus from *Tropocolum majus* root meristem. The chromosomes (ch), which are rather small compared to those of figure 3, occupy the peripheral portion of the nucleus; the remaining volume is filled with prenucleolar material. $\times 5,500$

FIGURE 5.—Micrograph of midtelophase nucleus from *Helianthus annuus*. Section $1\ \mu$ thick stained with azure B. $\times 6,000$

FIGURE 6.—Same nucleus as preceding figure photographed after digestion with ribonuclease. Large amounts of prenucleolar material have been removed between the chromosomes. $\times 6,000$

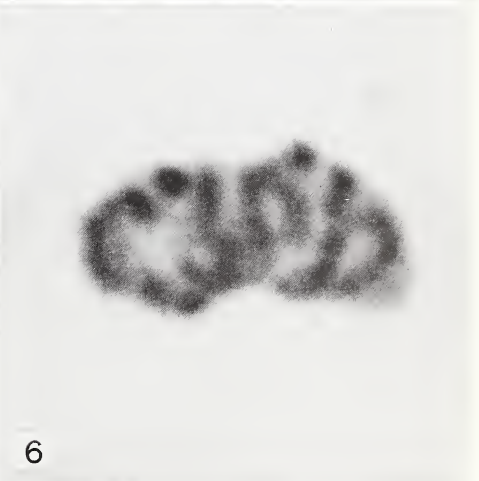
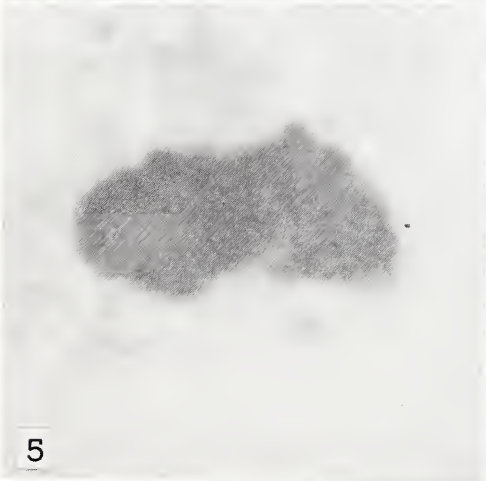
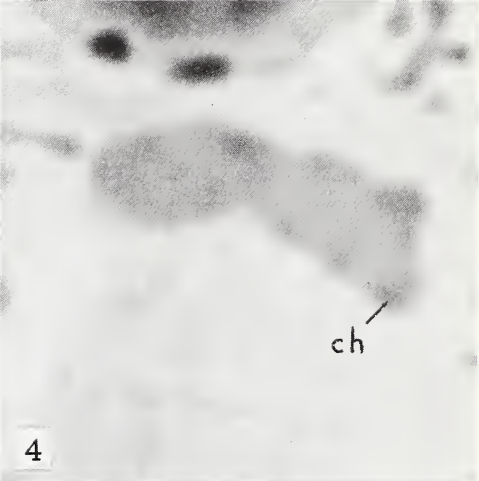
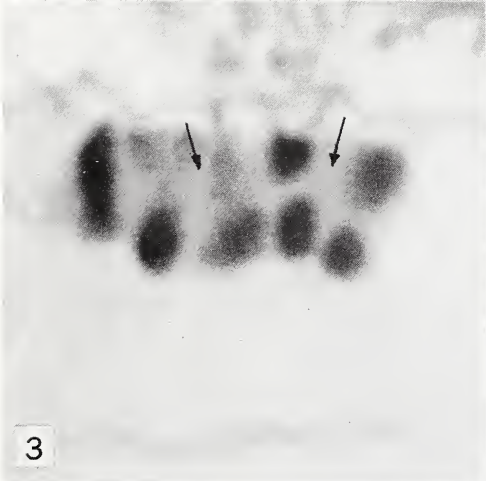
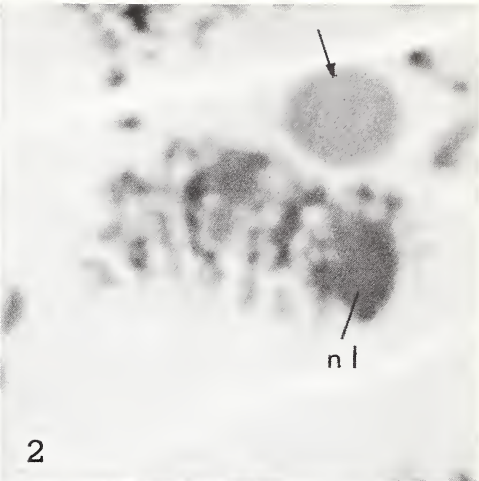
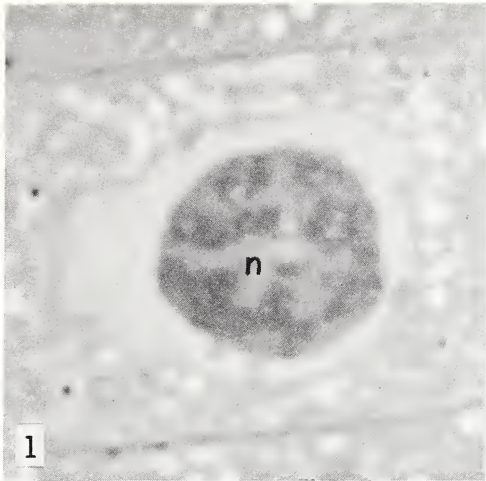
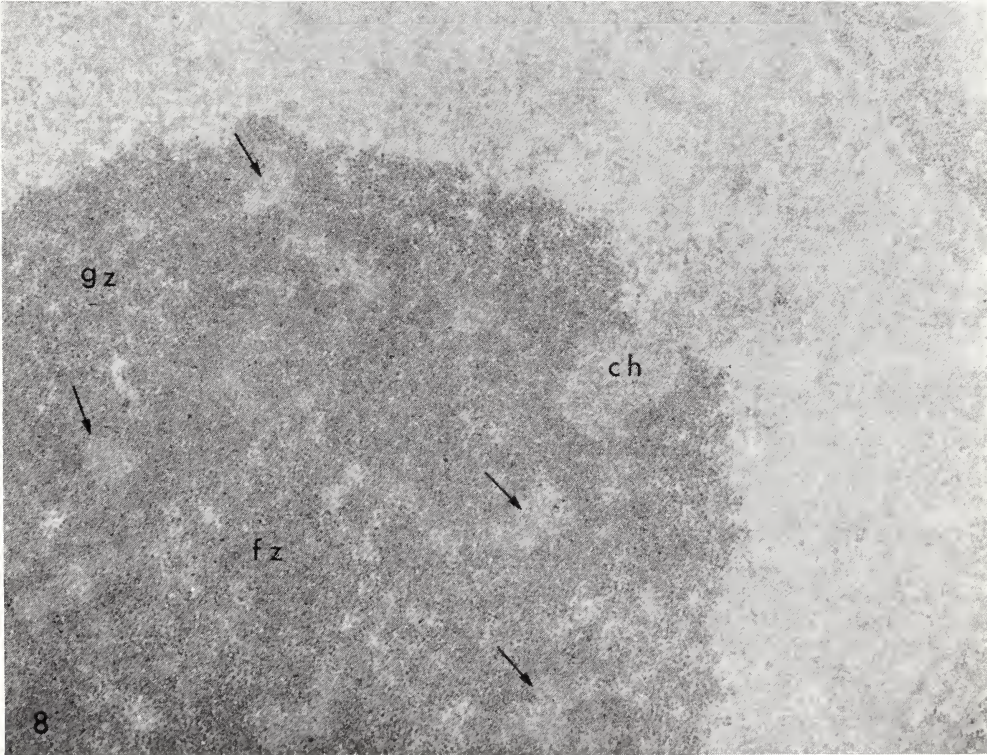
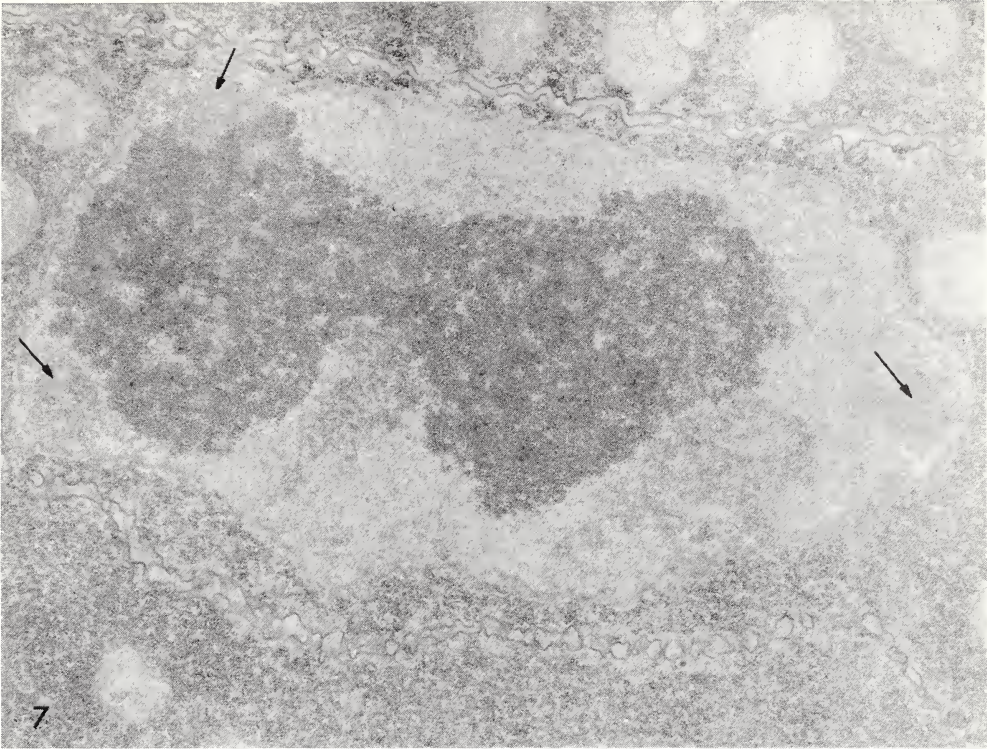


PLATE 12

FIGURE 7.—Electron micrograph of *Tropocolum majus* telophase nucleus. The nucleolus occupies the greater portion of the nuclear cavity. At that stage it consists of granular and fibrillar zones and also exhibits small vacuole-like areas. Most chromosomes (*arrows*) are margined. $\times 18,000$

FIGURE 8.—Portion of interphase nucleus from *Tropocolum majus* meristematic cell. A chromosome (*ch*) strand projects within the nucleolar mass. Besides the granular (*gz*) and fibrillar (*fz*) zones, the nucleolus also shows several lighter areas (*arrows*) characterized by a texture identical to that of the chromosomes. $\times 30,000$



Association of Nucleolus and Sex Chromosome in *Gryllidae* Spermatocytes^{1,2}

J. ROBERTO SOTELO and RODOLFO WETTSTEIN,
Instituto de Investigacion de Ciencias Biologicas, Departamento de Ultraestructura Celular, Avda Italia 3318, Montevideo, Uruguay

SUMMARY

Tridimensional reconstruction has been used to study the relationship of the nucleolus with the sex chromosome in the spermatocytes of *Gryllus argentinus*. The disposition of filaments similar to those forming the medial components of the synaptonemal complexes was studied, and the general morphology of the two bodies at prophase is shown in models made by superimposition of cardboard sheets cut according to the shape appear-

ing in each electron micrograph. Counts of the paired autosomes were made by the synaptonemal complexes traced in serial sections. In this way it was possible to determine the haploid number of the species ($n=14$) and to recognize the components which integrate the associated nucleolus and sex chromosome.—Nat Cancer Inst Monogr 23: 77–89, 1966.

ASSOCIATION BETWEEN nucleolar material and chromosomes has been observed to occur in the meiotic cycle in many species. Several cases of close association between the sex chromosome and the nucleolus of *Gryllidae* spermatocytes were reported as early as the beginning of this century. Difficulties caused by a lack of specific stains and the limited resolution of the light microscope impaired their study. Nevertheless, the investigations by Winiwarter in *Gryllotalpa* (1) are very accurate.

The association we are dealing with is particularly striking because of the complexity of the phenomena taking place during its development. These phenomena have been studied in this laboratory since 1959 and occurred in at least three species of *Gryllidae*. Moreover, a similar group of findings was reported in 1964 by Schin (2) who studied one European species.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function. Montevideo, Uruguay, December 5–10, 1965.

² Research partially supported by the Rockefeller Foundation grant RF-61034, and by Public Health Service grant GM-08337 from the National Institute of General Medical Sciences.

The association begins at the early prophase of the first spermatocyte and continues along the cycle, through divisions I and II and during spermatid maturation. The development of the complex body resulting from this association has been reported extensively, but the description was based on low magnification pictures taken from randomly mounted sections (3-5). We have studied the relationship with tridimensional reconstruction from serial sections and high magnification electron microscopy in an attempt to understand the mechanism by which the association takes place and the order of appearance of the various structures involved.

MATERIALS AND METHODS

The species studied were *Gryllus argentinus*, *Myogryllus verticalis*, and another species of *Gryllus* which has not been determined.

Fixation was performed in the standard way: The fixative was dropped *in situ* and the test tubes were removed afterward. The fixative was osmium tetroxide dissolved in Veronal buffer and the embedding medium was Araldite. Sections were made with a Porter-Blum microtome and examination was carried out in a Siemens Elmiskop I.

Sometimes tridimensional reconstructions were made by superimposition of transparent celluloid sheets alternatively with cardboard, the celluloid representing the chromosome part and the cardboard the nucleolar part of the complex. Other times only cardboard sheets were used. Schematic drawings complete the illustrations of the paper.

OBSERVATIONS

In the early spermatocytes the nuclear envelope encircles most of the chromatin mass which was identified as the sex chromosome (text-fig. 1). At this stage of the relationship between the nucleolus and the sex chromosome and the ordering in pairs of the homologous chromosomes has already taken place, as demonstrated by the presence in the nucleus of tripartite or synaptinematic complexes, but the chromatin along the synaptinematic complexes is diffuse and the nucleolus is small. The next step in the development of the spermatocytes is marked by the appearance of numerous chromomeres along the paired autosomes. Filaments of the order observed in the medial space of the synaptinematic complexes are at this time observed within the associated nucleolus and sex chromosome. The reconstructed nucleoli shown in the models (fig. 1) have already passed through the stage in which filaments have appeared inside them. The association with the chromosome is increasingly complex. From this stage on, three separate elements are found: (a) the tripartite groups traversing both the chromosome and the nucleolus; (b) the tubular com-



TEXT-FIGURE 1.—Schematic drawing showing the different stages of the associated nucleolus and sex chromosome during the meiotic cycle. 1) The nucleolus (*striped zone*) and the sex chromosome (*dotted zone*) are at separate places in the spermatogonia; 2) both components join at the beginning of pachytene; 3) and 4) they become linked by a synaptinemal-like complex at midpachytene; 5) tubular components and single filaments are in the nucleolar mass at about the same stage; 6) a complex array is at the end of pachytene; 7) the same complex is present at divisions I and II (A, autosome, S, complex); 8) and 9) the complexes are in the maturing spermatids.

plex of filaments within the nucleolar material, and (*c*) the single filaments (text-fig 2).

In some cases two or more synaptinemal-like structures traverse the nucleolus and chromosome mass and at least one of them is always seen. The main characteristic of these structures is that the nucleolar material occurs on one side of the medial component and the chromosome material occurs on the other (fig. 2). These linear arrays may start from separate places on the nuclear envelope and join after having traversed the main mass. A single tripartite structure then follows up from this point to a place on the nuclear envelope opposite its origin. Components of this kind were found in a series of 80 sections made with the purpose of counting the total number of synaptinemal complexes. The count was coincident with the haploid number of the species ($n = 14$), plus these elements.



TEXT-FIGURE 2.—The diagram shows the nucleolus and the chromosome as observed in part of a series of sections of a primary spermatocyte. The tubular component appears transversally cut in the horizontal section and longitudinally in the vertical one. The lines between the chromosome material (*dotted zone*) and the nucleolar material (*striped zone*) represent synaptonemal-like complexes.

One or more cylindrical tubes formed by elements showing the same pattern of structure as those mentioned previously are found within the nucleolar substance. In the axis of each cylinder there is a long thread of chromosome-like material (fig. 3). These components many times form a long rod extending into the nucleus but in other cases are partly included in the nucleolar mass.

Single filaments may occur in several places within the nucleolar mass. The degree of complexity they can reach cannot be estimated from the limited number of cases shown here, but previous observations have demonstrated that they increase with the advance of prophase. In random sections, it was found that they are usual components of the association. Their structure always corresponds to the structure of the medial component of the synaptonemal complexes but surrounded on all sides by nucleolar material.

DISCUSSION

As a contribution to the discussion of this paper we can advance only the following points of comparison.

The homologous chromosomes are associated at synapsis by a filamentous material that follows a pattern constant for all the autosomes of the species concerned. The pattern of *Gryllus argentinus* is shown in figure 3.

Meyer (6) recently suggested that these filaments may play a role in the process of recombination. He compared the number of filamentous bridges and the total number of genes per large autosome of *Drosophila* and considered that they match.

It has been observed that the nucleolar material is linked to the sex-chromosome material by a filamentous structure similar to that joining homologous autosomes. Whether we can consider this linking material as functioning in the way suggested by Meyer is an open question. In a recent paper Schin (7) suggested that parts of the sex chromosome of *Gryllus* are homologous and that intrachromosomal pairing occurs. Also, we must recall that elements of the same kind and disposition are found in the spermatid.

RESUMEN

En una etapa temprana de la profase meiótica (espermatocitos de *Gryllus argentinus*), el nucleolo y el cromosoma sexual se asocian para formar un solo cuerpo.

Esta asociación se caracteriza por la presencia de complejos sinapteinémicos que enlazan los materiales de ambos cuerpos. El componente medial de los complejos posee una estructura similar a la de los complejos de los autosomas.

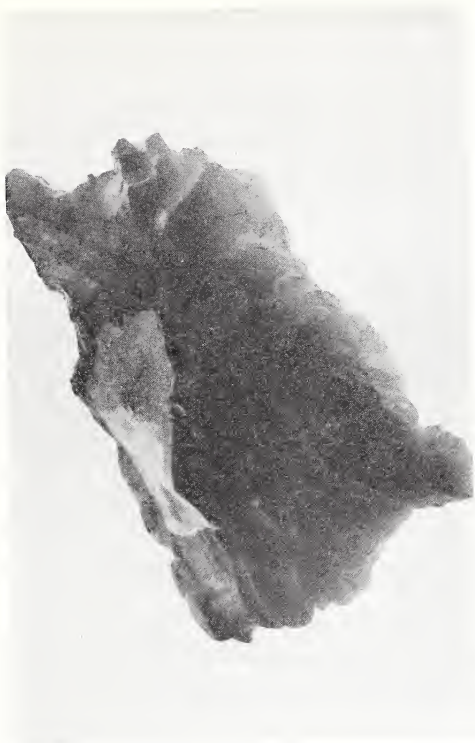
El cuerpo "nucleolo-cromosoma" se estudió por el método de reconstrucción tridimensional, se prepararon varios modelos constituidos por hojas de cartón sobrepuentats. La disposición de los componentes de tipo sinapteinémico se estudió en los mismos cortes o en otros similares. Adicionalmente, se estudió en los cortes seriados el complemento cromosómico de la especie ($n=14$) los que se ilustran por medio de un esquema.

REFERENCES

- (1) WINIWARTER, H.: Etude du cycle chromosomique chez diverses races de *Gryllotalpa gryllotalpa* (L). Arch Biol (Liege) 37: 515-572, 1927.
- (2) SCHIN, K. S.: Meiotische Prophase- und Spermatidenreifung bei *Gryllus domesticus* mit besonderer Berücksichtigung der Chromosomenstruktur. Z Zellforsch 65: 481-513, 1965.
- (3) SOTELO, J. R., and TRUJILLO-CENÓZ, O.: Electron microscope study on spermatogenesis. Chromosome morphogenesis at the onset of meiosis (cyte I) and nuclear structure of early and late spermatids. Z Zellforsch 51: 243-277, 1960.
- (4) ——— Electron microscope study on chromosome structure during meiosis. Path Biol (Paris) 9: 762-768, 1961.
- (5) SOTELO, J. R., and WETTSTEIN, R.: Electron microscope study on meiosis. The sex chromosome in spermatocytes, spermatids and oocytes of *Gryllus argentinus*. Chromosoma (Berlin) 15: 389-415, 1964.
- (6) MEYER, G. F.: A possible correlation between the submicroscopic structure of meiotic chromosomes and crossing over. In Proc 3d European Regional Conf Electron Microscopy. Prague, Czechoslovakia, Publishing House of the Czechoslovak Academy of Sciences, 1964, pp 461-462.
- (7) SCHIN, K. S.: Core-Strukturen in den meiotischen und post-meiotischen Kernen der Spermatogenese von *Gryllus domesticus*. Chromosoma 16: 436-452, 1965.

PLATE 13

FIGURE 1.—These photographs show 3 of the models obtained by serial reconstruction. The *white* surfaces represent the chromosome component, the *dark gray* ones the nucleolar component. The 2 figures *above* are different models. Those seen *below* are opposite faces of the same model.



1

PLATE 14

FIGURE 2.—Synaptonemal complex observed in the association of nucleolus and sex chromosome at the beginning of pachytene. The *left* side of the complex is chromosomal and the *right* is nucleolar.

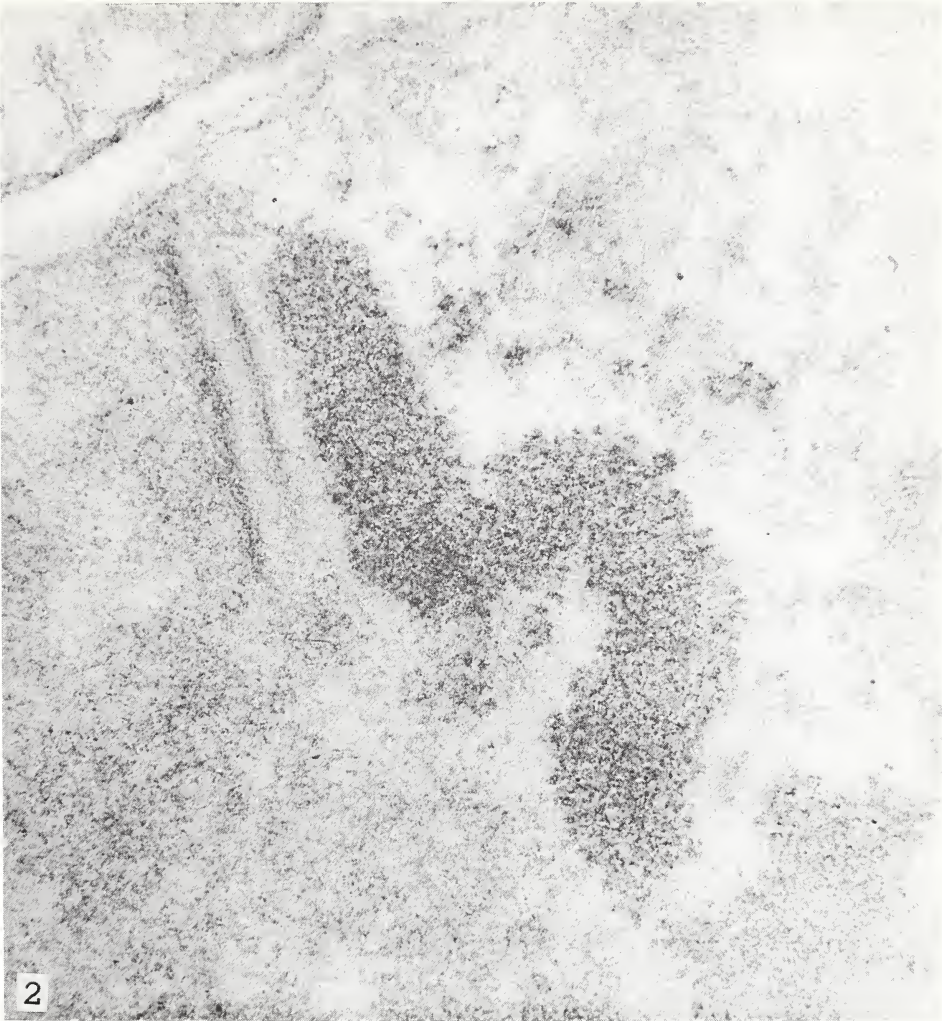
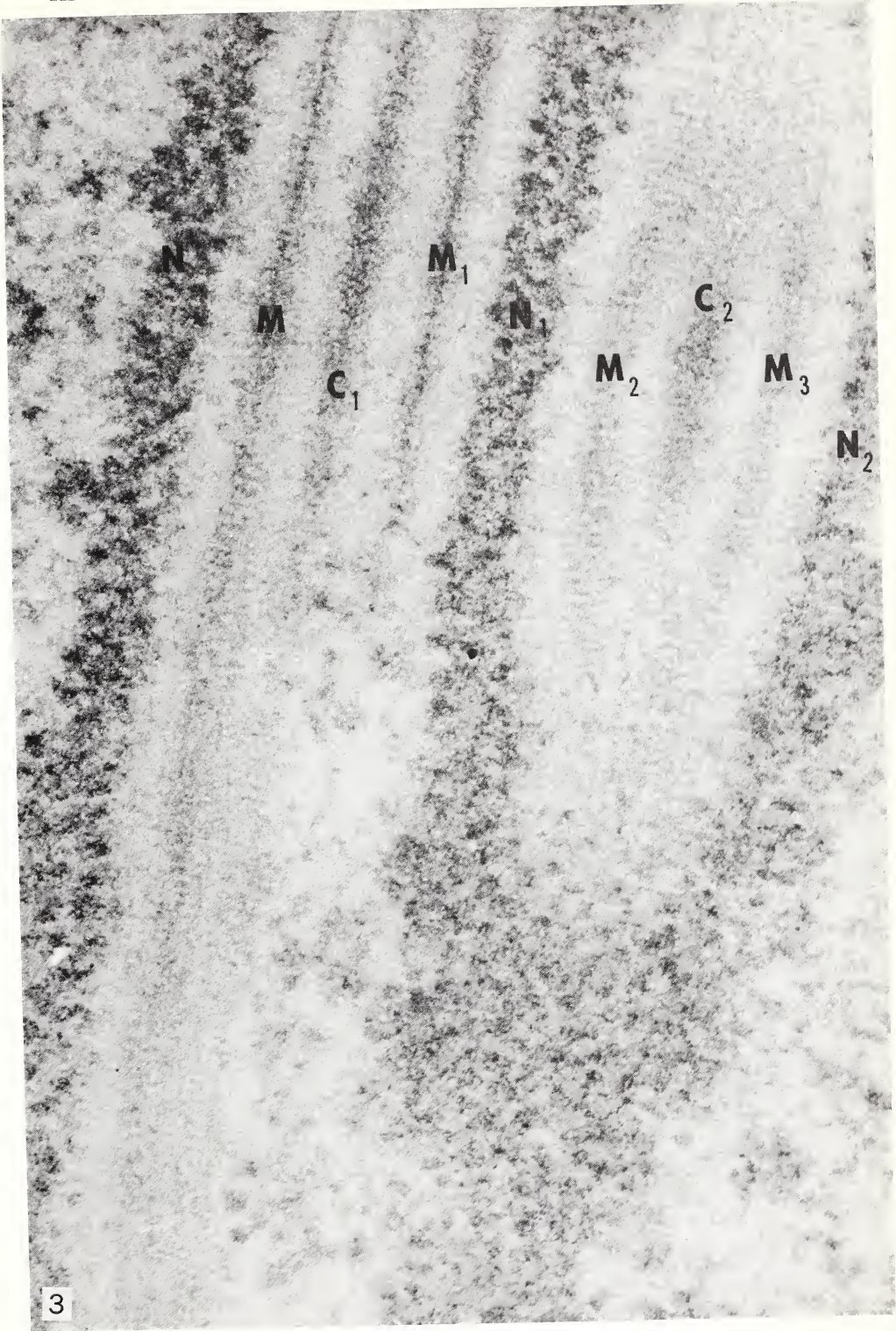


PLATE 15

FIGURE 3.—In this electron micrograph only a small part of the tubular component described in the text is illustrated. The tubular component is double in this particular association. The pattern of structure of the element marked M₁, M₂, and M₃, is similar to the one found in the pairings complexes of the autosomes of this species. N, N₁, N₂: nucleolar material; C₁, C₂: chromosomal material.



DISCUSSION

Wallace: Dr. Sotelo, I saw something in 2 of your electron micrographs which resembled sperm tails to me. Can you assure me that this is impossible?

Sotelo: Yes. The structure is completely different from the sperm tail. As you know, the sperm tails have tubular structure, with 9 tubular elements in the periphery and 2 in the axis. But the filaments that form the nucleolus and sex chromosome complex are filaments of the same kind seen in the medial components of the synaptonemal complex. That is, they are filaments similar to those uniting paired homologous chromosomes. These are the filaments which Meyer thinks may be related to the process of crossing-over (Proc European Regional Conf on Electron Microscopy, Delft II, 951-954, 1960).

Schultz: I should like to ask if the picture Professor Sotelo has been describing might not be accommodated by a scheme that has just occurred to me. I don't know whether it is bizarre or reasonable, but let me spell it out. What one must suppose, presumably, is that in the *Gryllus* X there exists a nucleolus organizer, and, in addition, that there are other regions of the X chromosome undergoing the synaptic process. *Gryllus* is XO; it has only one X chromosome. Therefore, what this might mean is that you have an association of regions of the X which are duplicated. In other words, the X could be an isochromosome of some kind with the nucleolar organizer close by. In this way one would find a synaptonemal complex between the homologous regions of the X. The way in which the X chromosome of Orthoptera behaves in the early meiotic prophase, with the ends of the X coming around to form part of the bouquet, has always been one of the peculiar things in the older cytological literature. I wonder whether you may not have provided a striking answer to this phenomenon. Would you find this entirely unreasonable?

Sotelo: At the beginning of the prophase after the last spermatogonial division one may find the nucleolus in one place and the sex chromosome in another place in the nucleus. A little later they come together, but at that time they are not linked by filaments even though synaptonemal complexes are already present in the nucleus. The chromosomes have started to pair and at this stage the nucleus and the nucleolus are still relatively small. But when one looks at a larger prophase nucleus which is in an already advanced pachytene phase, then one sees the nucleolar body with the chromosome mass in close contact with it and also the filaments coming from the nuclear membrane and linking the two materials. They make several revolutions within the mass and I don't know yet whether the tubular part is formed by these elements or in another way. We can distinguish three components in the early nucleolar complexes—the one similar to the synaptonemal complex, the tubular-like element, and another single element running inside tunnels within the nucleolus and similar to the medial component.

Schultz: It may be that the point Dr. Wallace was making and the one I was suggesting are in a sense related because we don't know too much about the relation of the components of the synaptonemal complex to the kinds of filamentous elements that constitute such things as cilia and flagella. And the question you have raised by these observations would either indicate that there are homologies within the X which make it pair with itself, or that there are other structures involving the nucleolar organizer which secondarily associate with the X.

Sotelo: From Moses' observation of the synaptonemal complexes, those made by treating with enzymes (Coleman and Moses, J Cell Biol 23: 63-78, 1964), we know that the elements of the medial components are protein in nature and have nothing

to do with DNA. It is possible that proteins may adopt a pattern of union between elements of different kinds and the existence of them in this particular association doesn't mean there is typical pairing.

Schultz: I brought this point up because it illustrates something that has been coming up all day, namely, that each species has its own peculiar genetic pattern and that this genetic pattern is going to determine to a large extent the morphology of the nucleolar formation and nucleolar behavior. I think we have seen perhaps more instances than we have realized of just these differences. We have been comparing mammalian cells from very different tissues; we have been comparing plant cells; we have just been comparing nucleoli in spermatocytes, and Dr. Swift showed us the enormous nucleoli from polytene chromosomes. If one were to adopt the very simple hypothesis, for which I have a great deal of bias, that the elementary nucleolar fibril was a loop of the type we know from the lampbrush chromosome, multiplied many times, consider how many different ways these loops could be wrapped up, and how many different aspects of them we would see in single sections. We have done some work with the *Drosophila* nucleoli in small cells that are quite a good deal simpler to analyze, and they do give some encouragement to this view (Ashton and Schultz, J Cell Biol 23: 7A, 1964).

Morphology, Structure, and Dynamics of the Nucleolonema¹

CLEMENTE ESTABLE, *Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay*

SUMMARY

The morphology, structure, and dynamics of the nucleolus are analyzed in a variety of cells by means of impregnation, staining, and observation of fresh material. The nucleolonema has been isolated: (a) by mechanical means (disruption of cells and nuclei); (b) in hypotonic solution; (c) by chemical means, using proteolytic enzymes. The nucleolonema is less attacked than the other nuclear structures. The chemical composition of the nucleolonema is different from that of the *pars amorphica*. It is concluded that the nucleolus is constituted by molecules organized in structures, the *nucleolonema*, and by molecules not organized in structures, the *pars amorphica*. In the latter, the substances result-

ing from the metabolism of the nucleolonema are located. An *intracellular internal medium* related to growth, multiplication, and restoration of the cell structures is postulated. The terminal growth of the nucleolonema, its self-reproduction by bud formation, as well as the permanence of some of its parts throughout mitosis also are postulated. An attempt to coordinate the principle *omnis nucleolonema e nucleolonema* with the existence of the chromosome nucleolar organizer is made. The behavior of the nucleolonema during some human and animal diseases, like syringomyelia and a chick disease called "Hutt's disease," is described.—Nat Cancer Inst. Monogr 23: 91-105, 1966.

OUR FIRST observations concerning the structure of the nucleolus were presented at the First International Congress of Biology held in Montevideo in 1930 (1). Reports of the filamentous and glomerular structure of the nucleolus were published (2-4) 20 years later. Almost at the same time, the studies by Borysko and Bang (5) and those by Bernhard, Hague-nau, and Oberling (6) confirming the existence of nucleolar filaments were reported. Lettré (7, 8) was one of the first researchers who referred to the structure of the nucleolus. But among the many publications that followed, we will only consider here those concerned with the interpretation of the facts as they relate to our basic concept that the nucleolus is essentially formed of molecules organized in structures—the *nucleolo-*

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5-10, 1965.

nema—and molecules not organized in structures—the *pars amorphia*.

The present report will be ordered as follows: 1) demonstration of the existence of the nucleolonema; 2) demonstration of the existence of the *pars amorphia*; 3) some information about the nucleolonema in pathology; 4) origin, reproduction of the nucleolonema, and its behavior during mitosis; 5) final comments.

DEMONSTRATION OF THE EXISTENCE OF THE NUCLEOLONEMA

It has been suggested that the nucleolonema is an artifact, a rosary of granules agglutinated by an excess of silver impregnation. If so, the true structure of the nucleolus would then be spherules and vacuoles. But before we accept this concept as definitive, the following fundamental questions should be answered: How can we estimate overimpregnation, overstaining, underimpregnation, or the exact point of impregnation? A previous knowledge of the nucleolar structure would provide the right reference to decide when the silver impregnation is excessive, insufficient, or correct, but how can this point be determined if the structures we are dealing with are not yet well known?

The best criterion of the adequacy of a staining technique is to compare its results with observations *in vivo*, but this is not always feasible. In the case of the submicroscopic structures such a comparison is beyond our means. The structure may be separated from the function only after death of the tissues and it is only in living beings that a close relation among structure and function, *functional structure*, exists. The comparison of the results of several different techniques complements the observation *in vivo*. Thus, the nucleolonema was studied *in vivo*, by staining techniques and by silver impregnation (figs. 1 through 5).

To obtain all possible information to support our former research, nucleolar isolation was carried out in isotonic and hypotonic solutions as follows: (a) without fixation or staining (fresh material); (b) after staining without previous fixation; (c) fixing the material to observe the actual action of the fixative reagents. In addition, small blocks or pieces of organs, previously impregnated *in toto* and dehydrated, were mounted in Canadian balsam, mechanically disrupted, then smeared, and examined by high-power objectives. Nucleolonema were also isolated by submitting the fresh material to the action of proteolytic enzymes such as those extracted from *Ficus carica*. By this procedure, the nucleolonema is more resistant to such enzymes than any of the components of the nucleolus or even of the nucleus so that the helical gyres of the nucleolonema can be easily seen.

The preceding techniques complement one another and reveal that the structure of the nucleolus is essentially composed of the *nucleolonema* and the *pars amorphia*. Were the nucleolonema an optical illusion provoked by a linear series of granules, the isolation of nucleoli in isotonic or hyper-

tonic media would bring about their spreading out (Brownian movement) which does not occur. We are *not* speaking of the components of the pars amorphia, which may contain granules, nor of the granular nucleolonemal buds from which new filamentous structures arise. The existence of granular corpuscles in the karyoplasm need not be discussed in relation to the structure of the nucleolonema.

Classical authors have described granules and vacuoles as regular components of the nucleolus. Cajal (9) determined their exact number and size in nerve cells: In the large pyramidal cells of the human cortex, the number of spherules varies between 24 and 36; in the medium ones between 16 and 24; in the small pyramids and in the short-axon neurons, between 4 and 8. Their diameter varies between 0.25 and 0.30 μ (in the small neurons they are less than 0.25 μ).

Admitting as valid that the nucleolus is of granular and vacuolar constitution and neglecting some technical details or the information obtained from microdissection, observation *in vivo*, etc., it would seem our knowledge of the structure of the nucleolus is definitive and research is at an end. Here we have a typical example of the Cajal aphorism: *no hay asuntos agotados, sino hombres agotados en los asuntos* (there are no exhausted subjects, but men exhausted of working on them).

The supposed existence of vacuoles in the nucleolus may be a mistaken observation or interpretation. It is an understandable mistake, because vacuole-like structures can be found when there are regions of uneven density in the pars amorphia and in the zones in which the nucleolonema turning over itself surrounds less dense micro areas of the pars amorphia.

Except for the spongelike nucleoli—some of which I have seen in electron micrographs taken by Sotelo—we have not observed nucleoli with vacuoles or alveoli as defined structures. As a matter of fact they can be considered as pseudostructures. It should be emphasized that the less dense zones (light or electron), limited by other zones of higher density, do not constitute special structures of the nucleolus. Fine vacuoles are limited by membranes distinguishable from the other surrounding structures.

The nucleolonema never loses its essential filamentous structure. Observed with the light microscope it appears as a granule when it is very small, but at higher magnifications and by careful turning of the micrometric knob its filamentous constitution appears clearly, as well as its two or more helical gyres (figs. 3, 4, and 5).

There are remarkable variations in the different cell types, as well as in the processes of growth and differentiation, cell activity, and pathological processes. In any of these conditions, changes can be seen in: (a) the number of filaments; (b) their length or diameter; (c) the amount of helical gyres; (d) the radius of the latter; (e) the size and density of the glomeruli. To have a precise and reliable concept about them, careful observation by means of adequate impregnation or staining techniques *in vivo* have to be carried out.

Herich (10, 11) recognizing the existence of the nucleolonema as a real structure believes the term "nucleolonema" is not adequate, because it is not constituted by a single substance. This is an acceptable rule in chemistry but not applicable to cytology. If we are to follow such a criterion, the name of any cell structure would be incorrect because any structure is a complex of many substances. Up to now we have contributed evidence demonstrating the existence of the nucleolonema.

The method of Wachstein and Meisel (12) for the demonstration of adenosinetriphosphatase is positive in the nucleolonema and negative in the pars amorpha. This would indicate that both components have a different enzymatic content (Reissenweber, unpublished data).

DEMONSTRATION OF THE EXISTENCE OF THE PARS AMORPHA

We have the pars amorpha defined as composed of "molecules not organized as structures," the word "structure" having only a cytological meaning.

We could designate the pars amorpha as the matrix in which the nucleolonema is embedded, but we do not think that it forms nucleolonemas. Pars amorpha always coexists with the nucleolonema but never occurs alone. A new extension of the Claude Bernard concept (13) on the internal milieu can be made by saying that there is also an intracellular milieu. The need of distinguishing the cell structures from the substances in which they are immersed, including those resulting from their metabolism, leads to this conclusion.

The relationship between nucleolonema and pars amorpha is of metabolic nature and accords with the concept mentioned above that the pars amorpha would be the internal milieu for the nucleolonema. There is no doubt that the substances corresponding to the nucleolar metabolism are included in the pars amorpha, either anabolites or catabolites. We lack tests that discriminate, either by light or electron microscopy, whether the whole pars amorpha is, with regard to the nucleolonema, a true internal milieu.

In the light microscope, bridges between the nucleolonemal gyres may be depicted. These bridges may be composed of small dense blocks of pars amorpha giving to the nucleolonema an anastomotic appearance. Electron microscopic research on the nucleolus is generally coincident with the facts discussed above. Figures 6, 6a, and 6b illustrate the fundamental observations on the nucleolonema and the pars amorpha.

SOME INFORMATION ABOUT THE NUCLEOLONEMA IN PATHOLOGY

Such distinguished researchers as Oberling and Bernhard (14) regarding the nucleolus of the tumor cells have said: "Les nucléoles sont de

nombre et de taille souvent exagérés, et ce fait représente un des caractères les plus constants de la cellule cancéreuse.”

Nucleolar pathology is not the subject of this Symposium, but the observations on pathologic nucleoli contribute to prove the existence of the nucleolonema as well as its polymorphism.

In a study on the pathology of syringomyelia (15) we observed some spirochete-like structures in the nuclei of hyperplastic ependymal cells (figs. 7, 8, and 9). The interpretations proposed at that time for these elements were: (a) spirochetes; (b) some alteration of the nuclear structure; (c) an alteration of the nucleolar shape; (d) Roncoroni's body (which is either an artifact or a folding of the nuclear membrane). At present we think this structure is a pathologic nucleolus.

By a careful examination of transitional stages and because microorganisms were never observed within the nucleus of any cell type and in any disease, the bacterial hypothesis was discarded. The spirochete-like transformation is indicative of a high degree of polymorphism as well as the filamentous nature and helical disposition of the nucleolus.

As a collateral digression, I call your attention to the well-known fact that except for virus and rickettsia, the nucleus and the nucleolus are always free of bacteria. We must know why it happens that way. This is an open question, but we are very far from the main subject.

Obvious changes of the nucleolonema also occur in the “loco o temblor congénito” (Hutt's disease) which is a disease of the chick leading to death before sexual maturation. The changes are different from those observed in the syringomyelia; in Hutt's disease the nucleolonema appears roughly outlined, dense, and irregularly shaped (figs. 10, 11, and 12).

The most difficult problem is whether the changes are primary or a consequence of the disease, and, moreover, to what degree they form part of the respective etiology.

ORIGIN, REPRODUCTION OF THE NUCLEOLONEMA, AND ITS BEHAVIOR DURING MITOSIS

During nucleolar neoformation in the nerve cell small spheres which later give rise to new nucleolonemas appear in the nucleus (figs. 13–18). These spheres are similar to the Levi-Barr corpuscle but of a different nature. The former are Feulgen-negative and the latter stain positively. On the other hand, the Barr corpuscle is a derivative of the sex chromosome and only exists in the female, whereas the spherules are a product of the nucleolonema and occur in both sexes. There is no apparent relationship between nucleolonemal corpuscles devoid of DNA or RNA described by Dutta *et al.* (16).

Have these spherules affinity with the “accessory body” noted in the nerve cells by Cajal? He describes them in the following way: “Se trata de un glóbulo de especiales propiedades, yacente en el carioplasma y

perfectamente separable por su tamaño, posición y capacidad tintorial, tanto del nucleolo principal y supernumerarios como de las granulaciones neutrófilas del jugo nuclear. En las neuronas voluminosas este corpúsculo es único, afecta forma esférica, muéstrase homogéneo, reside a cierta distancia del nucleolo y se caracteriza, en fin, por colorearse en presencia de la plata coloidal con un matiz diferente del tomado por el nucleolo o por teñirse cuando este último permanece incolorable. Su tamaño varía en las diversas células, siendo algo mayor que los gránulos argentófilos del nucleolo y muy superior a las finas granulaciones del carioplasma. Por término medio puede fijarse su diámetro en 0.5 de μ ."

In our concept, the accessory body described by Cajal is so similar to the nucleolar spherules related to nucleolonemal growth that one would conclude they are the same thing.

It is a fact that the nucleolonema is self-reproducing (figs. 13 through 18) and, undoubtedly, in the following cases every nucleolonema originates from another nucleolonema: (*a*) in the multiplication of the nucleolus without nuclear division; (*b*) during nuclear division without cell division; (*c*) during direct cell division (amitosis); (*d*) in mitotic cells characterized by nucleolar bipartition.

The analysis of the nucleolar mechanism is different in cells in which the nucleolonema is fragmented and scattered among the chromosomes. Even the nucleolonema recalls a chromosome before spreading out in minute fragments at the end of prophase.

When we refer to the nucleolonema persistence in all cells, and throughout mitosis, it does not mean that it should always take place in the same way. It has a proteiform morphology, but nevertheless it is built on a definite pattern and reproduces by buddings or germinal spherules.

The persistence of the nucleolonema is comparable to the persistence of the Golgi complex. In the germinal cells both persist throughout mitosis but not *in toto*; only the essential units we have described as *helicosomes* that are similar to the units described by Perroncito and named "dictyosomes" persist.

We think there is some coincidence between our interpretation and that of Lafontaine's (17, 18) in regard to prenucleolar material.

Nucleolonemal growth is always terminal and, for this reason, it is in some way comparable to the cone of growth of the nerve fibers, but with the nucleolonema each terminal spherule may separate from the main mass and give place to the formation of a new nucleolonema.

An extreme change occurs at mitosis when the nucleolonema becomes fragmented and disperses in such a way that it is difficult to trace it. In other cases, parallelism between nucleolonema and chromosome dynamics can be observed.

Any concordance between the concept of nucleolar organizer and nucleolonema persistence during mitosis is difficult. The self-reproducing properties of the nucleolonema cannot be ignored, although the total disappearance of the nucleolus at prophase, and its reappearance by genic

proteosynthesis must be admitted. It would be necessary to accept a double origin of the nucleolus, or a unified interpretation of the facts should be found. Facts are never contradictory. If one of two opposite theories is not false from inception, both theories may have points of contact and complement each other.

Which are the points of contact in this problem? We should emphasize the fact that fragments of the nucleolonema become scattered among the chromosomes at prophase and that part of them become attached to the latter. These fragments are similar to the germinal spherules which give rise to new nucleolonemas in other circumstances of the cell life. Admittedly, one of the fragments persists throughout mitosis and acts as a nucleolar organizer at telophase. On the other hand, the location of the nucleolus is not often a definite point along one determined chromosome. We must try to explain these exceptions which are more numerous than we expected.

A possible agreement between both concepts can be found in the investigations by *Hsu et al.* (19) using autoradiographic techniques. They conclude that there are cases in which the nucleolus is present in every phase of mitosis. This has been our contention for several years.

FINAL COMMENTS

Every reasonable interpretation of the nucleolus should account for the following facts: 1) the existence of the nucleolonema and of its reproducing properties; 2) the existence of the pars amorpha and its relationship with the surrounding medium; 3) the vesicular or alveolar appearance which may have one or the other or both components; 4) the granular appearance of the nucleolonema.

The general conclusion connoting that the nucleolus is composed of molecules organized in structures or nucleolonema, and of molecules not organized in structures or pars amorpha implies that infinite variations of the nucleolar aspects may be found. These variations may depend on the following factors: (a) increase or diminution of the total mass of the pars amorpha; (b) increase or diminution of the nucleolonemal mass; (c) increase or diminution of the density (light or electron) of the pars amorpha and of the nucleolonema (although the density of the former is lower than that of the latter); (d) increase or diminution of the number of the helical gyres and of their width.

Among the many aspects or appearances found in the nucleolar structure, which goes from the simplest spherule (generating bud) or from a single and short filament that tends to develop into a helical gyre to the complex glomerulus as big as a nucleus, the nucleolonema stands out as a fundamental structure.

A unitary interpretation of the nucleolar cycle may be conceived if the nucleolar organizer is identified with the reproducing part of the nucleo-

lonema as it can be demonstrated in the following conditions: (a) in the nucleolar multiplication without nuclear division; (b) in the nuclear division without cellular division; (c) in the direct division (amitosis); (d) in mitosis in which nucleolar persistence has been demonstrated.

We believe that a unitary interpretation may be more in agreement with the facts known than a dualist one. Therefore the general rule would be *omnis nucleolonema e nucleolonema*.

In this report, it was never stated that the nucleolonema is an autonomous structure because in the cell the functional synergy always depends on one or another component.

RESUMEN

Se analiza la morfología, estructura y dinámica del nucleolo en gran variedad de células, a favor de numerosas técnicas de impregnación, de coloración e *in vivo*.

Se aísla el nucleolonema a) por medios mecánicos; b) en soluciones hipotónicas; c) por medios químicos, recurriendo a enzimas proteolíticas que son más resistentes por el nucleolonema que por las otras estructuras nucleares.

Se concluye que el nucleolo está constituido por moléculas organizadas en estructuras, el *nucleolonema* y por moléculas no organizadas en estructuras, la *pars amorpha*, considerándose que en ésta se encuentran las sustancias propias del metabolismo del nucleolonema (existe un *medio interno intracelular* en relación con el crecimiento, multiplicación y restauración de las estructuras celulares).

Se prueba el crecimiento terminal del nucleolonema, su autoreproducción mediante brotes o yemas, así como la permanencia de elementos del mismo en todas las fases de la mitosis.

Se hace la tentativa de conciliar la fórmula *omnis nucleolonema e nucleolonema* con la existencia del *organizador nucleolar* cromosómico.

Se estudian comportamientos del nucleolonema en algunas enfermedades, como la siringomielia y en el pollo loco congénito, enfermedad que conduce a la muerte antes de la madurez sexual.

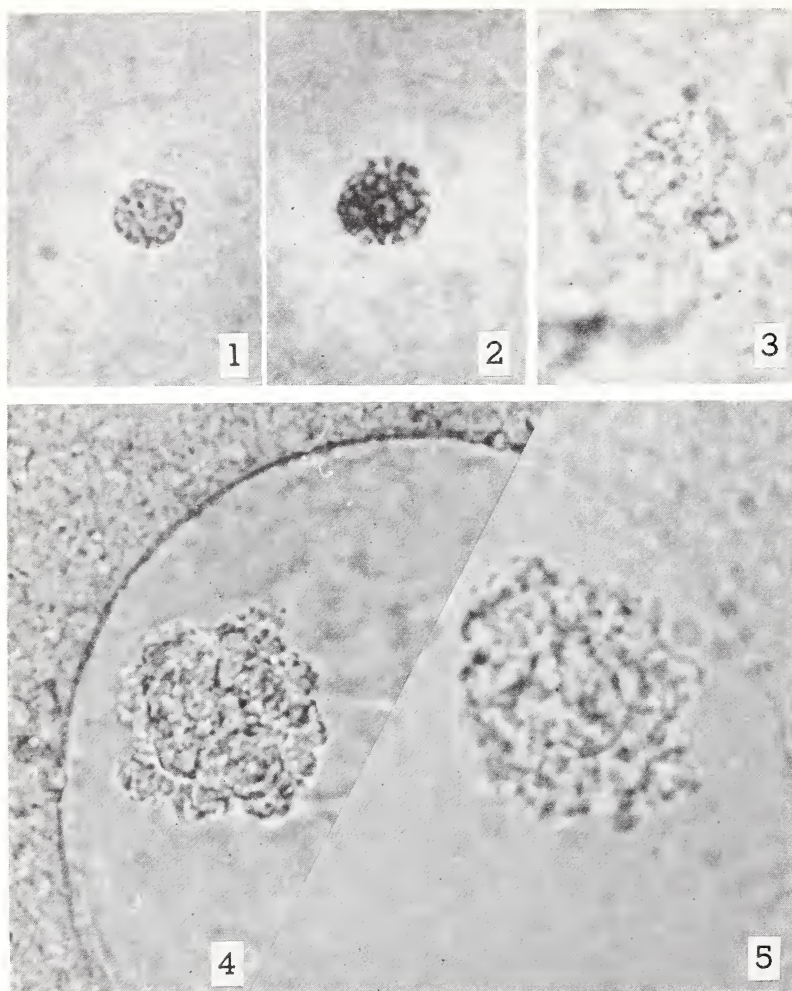
No encontrándose jamás en infecciones agudas o crónicas bacterias, tripanosomas, etc. dentro del nucleolo ni del núcleo—se exceptúan virus y *rickettsias*—se plantea el problema de si el nucleolo o el núcleo elaboran antibióticos o anticuerpos.

La comunicación fue ilustrada con un film.

REFERENCES

- (1) ESTABLE, C.: Estructura del nucleolo y algunas experiencias tendientes a demostrar su significación biológica. Primer Congr Int Biol y Congr Med del Centenario 9: 950-953, 1930.
- (2) ESTABLE, C., and SOTELO, J. R.: Una nueva estructura celular: el nucleolonema. Pub Inst Invest Cien Biol Montevideo 1: 105-126, 1951.
- (3) ———: The behaviour of the nucleolonema during mitosis. In *Fine Structure of Cells*. Groningen, P. Noordhoff, 1955, pp 170-190.
- (4) ———: Technical procedures for the study of the nucleolonema. *Stain Techn* 27: 307-312, 1952.
- (5) BORYSKO, E., and BANG, F. B.: Structure of the nucleolus as revealed by the electron microscope. Preliminary report. *Bull Hopkins Hosp* 89: 468-473, 1951.

- (6) BERNHARD, W., HAGUENAU, F., and OBERLING, C.: L'ultrastructure du nucléole de quelques cellules animales, révélée par le microscope électronique. *Experientia* 8: 58-63, 1952.
- (7) LETTRÉ, R.: Observations on the behavior of the nucleolus of cells in vitro. In *Fine Structure of Cells*. Groningen, P. Noordhoff, 1955, pp 141-150.
- (8) LETTRÉ, R., and SIEBS, W.: Beobachtungen am Nucleolus in vitro gezüchteter Zellen. *Z Krebsforsch* 60: 19-30, 1954.
- (9) RAMON Y. CAJAL, S.: El núcleo de las células piramidales del cerebro humano y de algunos mamíferos. *Trabajos del Lab de Invest Biol de la Univ (Madrid)* 8: 27-62, 1910.
- (10) HERICH, R.: New facts on the structure of the nucleolus. *Naturwissenschaften* 50: 625-626, 1963.
- (11) ———: The nucleolus structure. II. Differentiation, morphological build-up and development of nucleolar structures. *The Nucleus* 7: 59-66, 1964.
- (12) WACHSTEIN, M., and MEISEL, E.: Histochemistry of hepatic phosphatases at a physiologic pH. *Amer J Clin Path* 27: 13-23, 1957.
- (13) BERNARD, C.: Leçons sur les propriétés physiologiques et les altérations pathologiques des liquides de l'organisme. Paris, Baillière, 2 vol, 1859, 1004 pp.
- (14) OBERLING, CH., and BERNHARD, W.: L'ultrastructure de la cellule cancéreuse. In *Problèmes de Structures, d'Ultrastructures et de Fonctions Cellulaires* (J. André Thomas, ed.). Paris, Masson & Cie, 1955, pp 280-306.
- (15) ESTABLE, C., and ARANA IÑIQUEZ, P.: Sobre histopatología de lairingomielia y la causa de la disociación de la sensibilidad. *An Fac Med Montevideo* 26: 821-916, 1941.
- (16) DUTTA, CH. R., SIEGESMUND, K. A., and Fox, F. A.: Light and electron microscopic observations of an intranucleolar body in nerve cells. *J Ultrastruct Res* 8: 542-551, 1963.
- (17) LAFONTAINE, J. G.: Structure and mode of formation of the nucleolus in meristematic cells of *Vicia faba* and *Allium cepa*. *J Biophys Biochem Cytol* 4: 777-784, 1958.
- (18) LAFONTAINE, J. G., and CHOUNARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167-201, 1963.
- (19) HSU, T. C., ARRIGHI, F. E., KLEVECZ, R. R., and BRINKLEY, B. R.: The nucleoli in mitotic division of mammalian cells in vitro. *J Cell Biol* 26: 539-553, 1965.



FIGURES 1 AND 2.—The nucleolonemal glomeruli show some granular and vacuolar appearance. *Formatio reticularis*, medulla oblongata of the cat. Silver impregnation.

FIGURE 3.—*In vivo* photomicrograph of the nucleolonema from a salivary cell of *Armadillidium nasatum* B.L. This picture is one sequence of a film made to demonstrate the helical nature of the filament.

FIGURES 4 AND 5.—Nucleolonema glomeruli from oocytes of *Armadillidium nasatum* B.L. Examination in fresh material. The filaments and their helical gyres can be perceived.

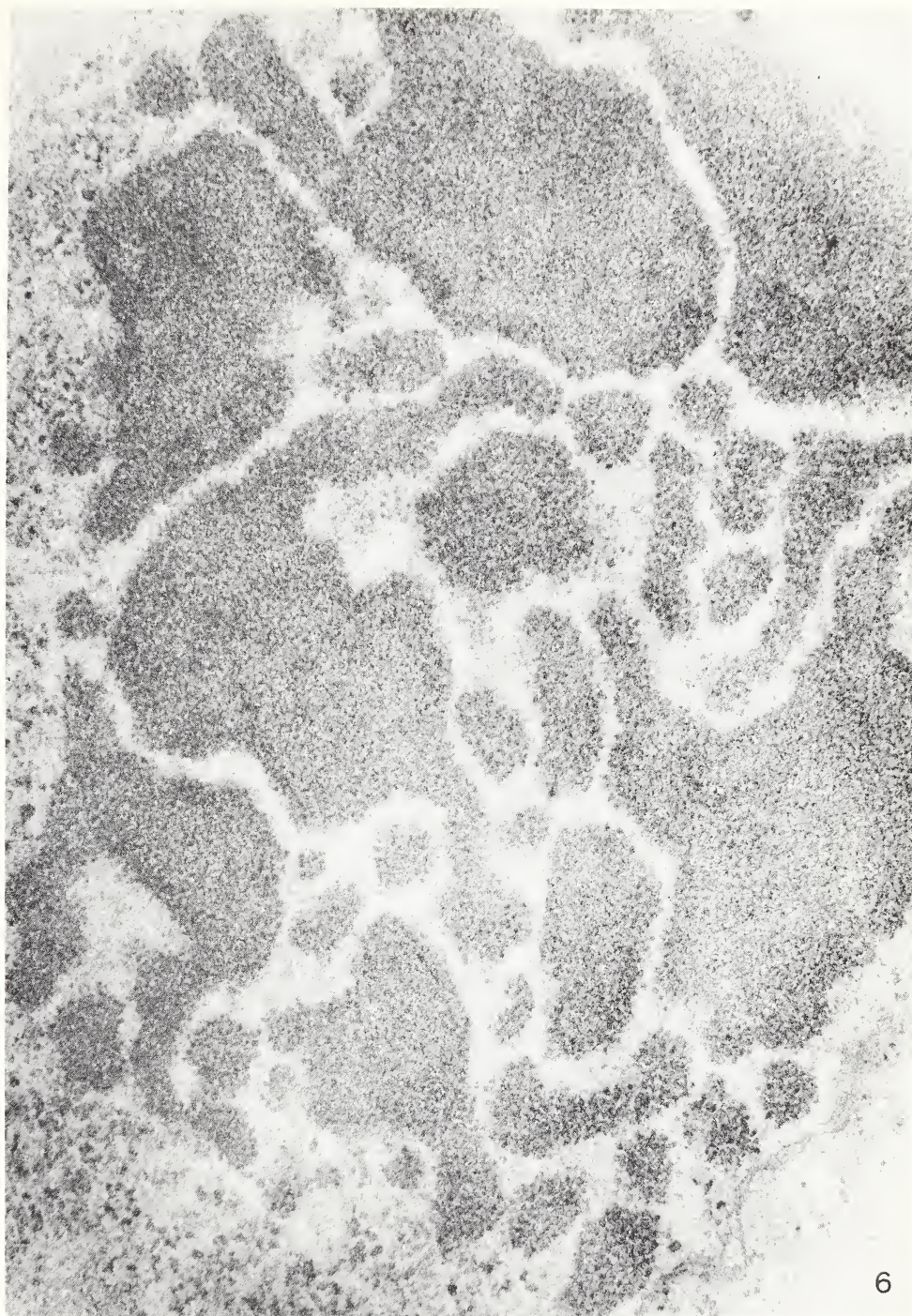
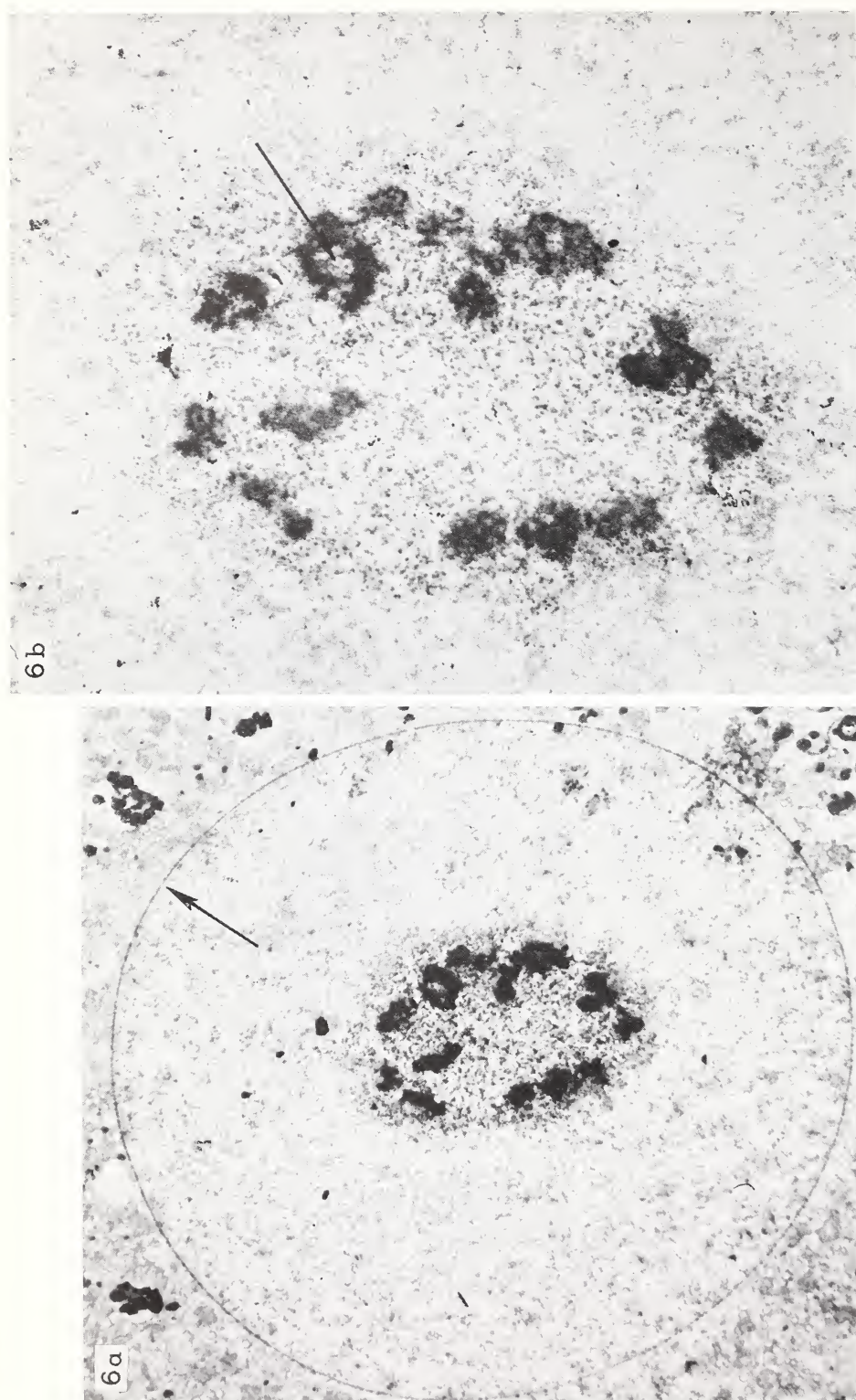
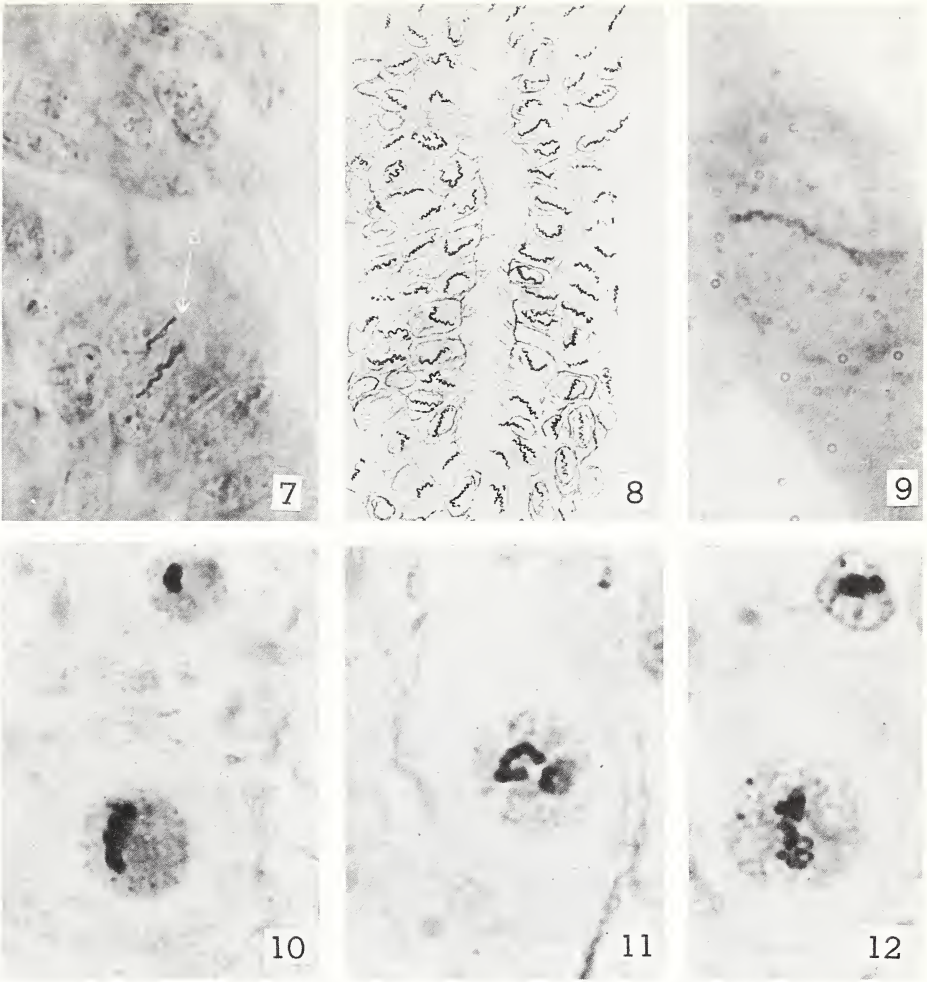


FIGURE 6.—Electron micrograph of the nucleolonema from a spermatocyte I of *Omezcachac servillci*, Blanch. No anastomoses are observed in this nucleolus.
× 60,000

Figure 6 photographed by J. Roberto Sotelo of our Institute.



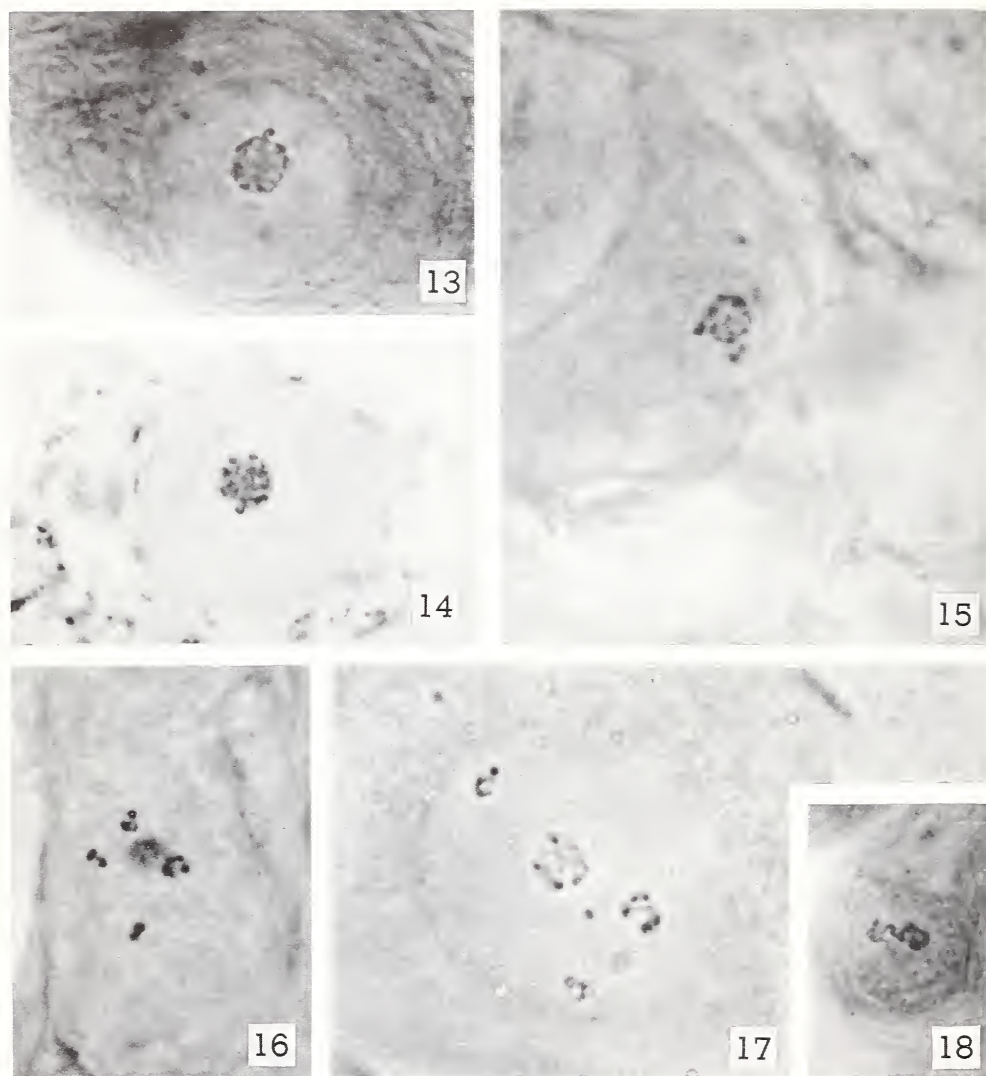
FIGURES 6a AND 6b.—Nucleus from a salivary cell of *Armadillidium nasatum* B.L. Note contrast between the nucleolus and *pars amorpha*. The nuclear membrane is signaled by an arrow. In 6b unitive bridges or anastomotic tracts of the helical gyres are seen (arrow). 6a. $\times 3,100$; 6b. $\times 8,000$



FIGURES 7 AND 8.—Pathological reaction of the nucleolus observed in the ependymal of the human spinal cord (syringomyelia). The nucleolonema shows a spirochete-like morphology.

FIGURE 9.—The spirochete-like nucleolonema shown in this picture can be mistaken with the Roncoroni rod or with a folding of the nuclear membrane.

FIGURES 10, 11, AND 12.—Pathological changes in Hutt's disease. The nucleolonema appears compact and shortened.



FIGURES 13, 14, AND 15.—Several stages of the developing of new nucleolonema by means of buds arising from the nucleolar glomerulus.

FIGURES 16, 17, AND 18.—Formation of new nucleolonemas from the budding spherules originated in the nucleolonema glomerulus. In some cases the new glomerulus remains attached to the original one.

DISCUSSION

Busch: Professor Estable, you mentioned that under certain conditions the *pars amorpha* is decreased in size. Could you define some of the conditions under which you see these changes in the *pars amorpha*?

Estable: The variations of the nucleolonema and the *pars amorpha* are very complex. In the latter, volumetric and density variations occur. In the former, polymorphism is characteristic, consisting of variations in the looseness or compactness of the helices or the number of nucleolonemal buds, *i.e.*, the terminal growths which give origin to new nucleolonemas. In some cases these buds separate from the original nucleolonema without being completely detached. The greatest changes are observed during cell growth and differentiation, or during cellular hyperactivity, regeneration, degeneration, or other pathological changes.

Love: Professor Estable, what are the cytochemical properties of the nucleolonema? Does it contain RNA, DNA, and protein, and does it vary under pathological conditions?

Estable: The histochemistry of the nucleolonema is studied in our Institute by some of my collaborators. Undoubtedly there is RNA in it. But with regard to DNA, we should not mistake the nucleolonema with the heterochromatin associated with the nucleolus. There is no doubt that the nucleolonema must contain enzymes like alkaline phosphatase and some not so well demonstrated, such as adenosinetriphosphatase.

Pavan: It was not clear to me, Dr. Estable, why you projected the figure of the work of Hsu (19) and commented about the nucleolonema or nucleolonemal material along the chromosomes. Could you clarify this point for me?

Estable: The problem posed is whether the substances composing the nucleolus persist or totally disappear during mitosis. I wanted to point out some relationships to the persistent nucleolonemal fragments throughout mitosis. The autoradiographic images of Hsu and collaborators are similar to some of our images. We do not say that the entire nucleolonema persists throughout mitosis nor that the fragments noted in mitosis become reconstituted. Our recent observations suggest that a small part of the nucleolonema is enough to generate new nucleolonemas and thus nucleolar reappearance takes place in telophase and interphase.

Lettré: Professor Estable gives us some difficulty by applying "nucleolonema" on the other hand to a filamentous structure which he can demonstrate within the nucleolus of living cells and on the other hand to material which can be demonstrated by silver impregnation, for example, in connection with nucleolar filaments during interphase or with numerous chromosomes during mitosis.

Morphological Observations on the Nucleolus of Cells in Tissue Culture, With Special Regard to Its Composition^{1,2}

RENATE LETTRÉ,³ WILHELMINE SIEBS,³ and NEIDHARD PAWELETZ,⁴ *Institute for Experimental Cancer Research of the University of Heidelberg, Heidelberg, Germany*

SUMMARY

This paper deals with observations on the filamentous structures in the nucleoli of fibroblasts and tumor cells in tissue culture under normal and experimental conditions. Light microscopy and electron microscopy were used. Under certain conditions depending on fixation, degree of flattening of the cells, and metabolic situation of the nucleolus, the filaments gave a positive Feulgen reaction. This characterized them as intranucleolar parts of chromosomes. The sometimes considerable length of the filaments contradicts the assumption that they represent only the secondary constriction of chromosomes. Unfolding of the nucleoli, e.g., by adenosine, helped in clearly demonstrating the filaments in electron as well as light microscopy. These structures were also successfully demonstrated by KMnO_4 and by silver impregnation. Occasionally, expanding

and retracting loops bordered the nucleoli. Indications of looplike formations, originating from the intranucleolar chromatin strands, could be found in stained preparations. Two chromatin strands sometimes formed a ribbon-like structure by interlacing loops, sticking together through the synthesized material. Obliteration of the Feulgen reaction in the central region but staining of the framing strands would explain the appearance of the "nucleolus-associated chromatin." According to our results we regard the "nucleolonema" as a complex formation, composed of different materials, its "backbone" being of chromosomal nature. Observations indicate that the functionally active regions of the intranucleolar chromatin may be present in the state of lampbrush chromosomes.—*Nat Cancer Inst Monogr* 23: 107–123 1966.

IN 1951 Estable and Sotelo (1) described the nucleolus as a cell organelle composed of amorphous material and a filamentous structure and designated it "nucleolonema." The special interpretation given to the nucleolo-

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This paper included a filmstrip entitled "Behavior of the Nucleolus of Cells Cultivated *In Vitro*."

³ Cytology.

⁴ Electron microscopy.

lonema excluded its chromosomal origin. In the following years the name "nucleolonema" was taken over, especially by the electron microscopists, without general acceptance of its original interpretation.

Soon after, Lettré and Siebs (2) began their investigations on the nucleolus—perhaps fortunately without knowledge of the work of Estable and Sotelo—thus having no prejudice with regard to the nature of the nucleolonema. Our interest in the nucleolus arose from the observation of a fascinating variability in its appearance in cells in tissue culture, especially so when they were exposed to a variety of environmental conditions. The appearance of the nucleolus proved to be a most sensitive indicator of the metabolic situation in the cell (3-8).

In accordance with Estable and Sotelo, Lettré and Siebs (2-8) found the nucleolus composed of a filamentous structure and of unstructured material. However, obtaining a positive Feulgen reaction of the filaments, we interpreted these structures as functionally active parts of chromosomes in contrast to Estable and Sotelo's interpretation.

"NUCLEOLONEMA," NUCLEOLAR CHROMATIN, AND "PARS AMORPHA"

The following terms are used for the morphological description of the structured components of the nucleolus:

The *nucleolonema*, regarded by most authors as not of chromosomal origin, is visible in most electron micrographs and is generally located inside of the nucleolus. In light microscopy it has been described only by a few authors [*e.g.*, Weissenfels (9)] since it cannot be detected in conventional material and by routine methods.

When referring to the chromatin component of the nucleolus, most cytologists think merely of a *nucleolus-associated chromatin* or of the *nucleolar organizer*, which both give the appearance of heterochromatic regions attached to the nucleolus.

Functionally active chromatin, that is, *euchromatin*, found to be present in the form of chromatin *lamellae*, has been demonstrated by Granboulan and Granboulan (10) by combining histochemical and electron microscopical methods.

Chromatin inclusions between the strands of the nucleolonema were observed by several electron microscopists [*e.g.*, Hay and Revel (11)]. These inclusions were occasionally interpreted as indentations of extranucleolar chromatin.

According to the biochemical models, "organizing" chromatin should be functionally active, that is, decondensed strands should be present at least partly as DNA/RNA hybrids. It could be expected that they are surrounded by material which might be partly discharged by the chromatin strands (RNA) which are partially synthesized around them (protein,

corresponding to the given RNA information). In addition, lipids could be expected to be present, interacting in these processes.

A rather high DNA content of nucleoli has been shown by biochemical methods [Monty *et al.* (12); Desjardins *et al.* (13)], and the presence of DNA could be confirmed with the use of ^3H -thymidine [Harris (14)]. Here, when interpreting the results, we have to consider carefully the interval between the addition of the label and the time of fixation. Chromatin which is functionally active probably would not be able to reduplicate at the same time, *i.e.*, to incorporate thymidine and vice versa.

There is evidence for the relation between the nucleolar DNA component and the synthesis of ribosomal RNA based on the formation of hybrids between these components [Ritossa and Spiegelman (15)], and there also is proof for the occurrence of protein synthesis in the nucleolus by use of labeled amino acids.

These results raise the question of the meaning of the association of the nucleolus with heterochromatin since this is defined as being inactive.

We will now present our observations and at the end of the paper discuss whether some of the seemingly contradictory findings mentioned can be brought into agreement.

Although we previously avoided the term "nucleolonema" because our interpretation of this structure differs from that proposed by Estable and Sotelo, we think that it is reasonable to use the term here since it is generally accepted. However, we must stress the point that *we regard the nucleolonema as a complex formation, composed of different material, its "backbone"—so to speak—being of chromosomal nature.*

Our interpretation explains the different aspects of the nucleolus as indicative of different quantitative and qualitative relationships between the nucleolonema and the rest of the nucleolar substance. These differences depend on the various cell species used and on the different actual metabolic situations.

If one assumes that the nucleolonema contains part of the nucleolar chromosomes, its aspect may vary depending on the following factors:

- | | | |
|---|---|--|
| A) <u>CHROMOSOMES CAN BE CONDENSED</u> | = | "inactive" chromatin |
| <u>Mitosis and Meiosis</u> | | <u>Interphase</u> |
| transport-form | | "repressed" chromatin
(heterochromatin) |
| B) <u>CHROMOSOMES CAN BE EXTENDED</u> | = | "active" chromatin
(euchromatin) |
| a) reduplicating | = | <u>DNA/DNA Hybrid</u> |
| b) <u>functionally active</u> | = | <u>DNA/RNA Hybrid</u> |
| <u>Meiosis (Dictyotene)</u> | | <u>Interphase</u> |
| lampbrush chromosomes | | "puff" formation
lampbrush form |
| C) <u>SYNTHESIZED MATERIAL</u> can be either accumulated around the chromosomal filaments or released into the nuclear sap depending only on the quantity and quality of the produced material. | | |
| D) <u>CHROMOSOMES HAVE THE CAPACITY OF PAIRING</u> as well in meiosis and mitosis as in interphase. | | |

There can be different stages of coiling, the extended chromatin representing the "active" chromatin; when it is functionally active it may be defined biochemically as being in the state of a DNA/RNA hybrid; its morphological appearance may be compared either to a puff formation in polytenic chromosomes or to the loop formation in lampbrush chromosomes.

The synthesized material can be either accumulated around the chromosomal filaments or may be released into the nuclear sap, depending on the quantity and quality of the material produced.

In general, these morphological details cannot be seen in interphase nuclei of cells of warm-blooded animals *in vivo*. How is it that our material reveals all these intranucleolar details? The simplest and probably the correct explanation seems to be that this is because the cells are flattened in tissue culture. The cells spread on the glass surface, their diameter frequently increasing twofold or even more. In many of the flattened nucleoli, a nucleolonema is visible, in living as well as in properly stained cells. Depending on the metabolic situation, the nucleolonema is more or less embedded in the unstructured nucleolar substance.

In the first plate an experiment is demonstrated which was extremely helpful in showing the relationship between the nucleolonema and the unstructured material and in giving more details about the nature of the nucleolonema. Hughes (16) described the effect of adenosine and other purine and pyrimidine derivatives on mitosis in cells in tissue culture. He observed what he calls "a breaking up of the nucleolus into separate granules," but did not comment on this observation. We repeated this experiment with many different cell strains and studied in motion pictures the "unfolding" of the nucleolus, or rather the "unmasking" of the nucleolonema under the action of adenosine. Figures 1, 2, and 3 are pictures of a living chicken fibroblast in which there is transition of the dense nucleolus to fine filamentous strands with some adhering droplets. Figure 4 is an electron micrograph of a chicken fibroblast after treatment with adenosine. We think that this is the first clear demonstration of distinct chromatin structures in interphase nuclei of warm-blooded animals. Figures 5, 6, and 7 show the same experiment in a HeLa cell, the last picture representing the Feulgen reaction of the unmasked nucleolonema.

The different aspects of the nucleolar structures in both cell types are mainly caused by the different arrangement of the chromatin strands: In the chicken fibroblast, single strands of a rosary-like appearance can be distinguished; some nucleolar material has persisted in the form of droplets. In the HeLa cell the strands are aggregated. Here nucleolar material is probably present as a fine coating of the strands. The Feulgen reaction is strongly positive.

IDENTIFICATION OF DIFFERENT COMPONENTS OF THE NUCLEOLONEMA AND OF THE PARS AMORPHA

Figures 8 through 27 are photographs of cells prepared by different methods for the purpose of identifying different components of the pars amorpha and of the nucleolonema. These are all HeLa cells, grown on coverslips in liquid medium. We were especially interested in the question of the location of lipids in the nucleolus. The next question was whether the silver-impregnation method [after Estable and Sotelo (1) or Gonzalez-Ramirez (17)] was specific for a component attached to the nucleolonema only, or whether it could also be found distributed in the nucleolar material.

Figures 8 and 12 are pictures taken after fixation with KMnO_4 . This fixation is believed to be specific for lipoprotein. We find no specificity in any particular cellular component but a more or less intense staining of different cytoplasmic, nuclear, and nucleolar structures. The nucleoli have a fine granular appearance. No surrounding nucleolar material is discernible.

Figures 9, 10, and 11 correspond to figures 13, 14, and 15, the former being "fully printed" and the latter being "underprinted"; that is, the development of the photographs was interrupted early, so that the intranucleolar structures were more clearly revealed. Figures 9 and 13 show a staining for lipids by Berenbaum's method (18). Both the pars amorpha and the nucleolonema are stained. When underprinted, these pictures reveal structures similar to those seen after KMnO_4 fixation. Their texture is reminiscent of the "honeycomb-like" structures that Granboulan and Granboulan (10) demonstrated at the surface of some nucleoli. Figure 9 is very similar to a picture published by Suskind (19) of a HeLa cell stained with Biebrich scarlet; his experiments suggest that the sites of ribonucleoprotein synthesis may be demonstrated by this method. Our results suggest the presence of lipids in addition to ribonucleoprotein in the nucleolar material as well as coating of the chromatin strands.

Figures 10 and 14 demonstrate the silver-impregnation technique of Gonzalez-Ramirez (17) after fixation with glutaraldehyde. Figure 10 shows a lightly stained nucleus, moderate coloration of the amorphous material, and strong black impregnation of some material, which is arranged like beads on a string.

Figure 11 shows the same impregnation following fixation with formalin. Here the nucleus remained unstained and no pars amorpha could be detected, but the droplets of strongly impregnated material were again arranged in the form of a beaded string. When unfolding the nucleoli by a pretreatment with adenosine, we obtain pictures that correspond very well to the looser parts of both nucleoli in figures 14 and 15.

Figures 16 through 19 show untreated HeLa cells fixed according to Serra's technique and then subjected to the Feulgen reaction. The nuclei

in figures 17 and 18 seem to belong to a higher range of ploidy. Such nuclei show the strongest Feulgen reaction of the filaments when the latter are paired. In figures 16, 17, and 19 the pairing and coiling are easily discerned.

Figures 24 through 27 show nuclei of the same kind of cells stained with hemalum. The photographs of the same cells (figs. 20, 21, 22, and 23) are underprinted to make the nucleolonemas more visible. The underprinting gives an indication of the underlying chromatin structures, although the strands are much coarser because they are coated with adhering material.

LOOPLIKE FORMATIONS OF THE NUCLEOLONEMA

Already in 1954 Gall, according to Vincent (20), suggested the analogy between lampbrush loops and the nucleolonema. Our following observations point in this direction.

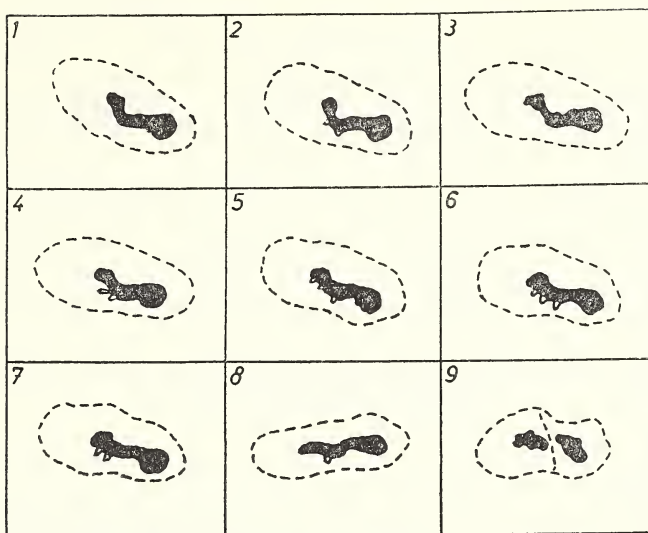
In figures 24 through 27 fine strands radiating in a right angle from the axis of the nucleolonema are clearly visible. In figures 20 through 23 they are hardly visible, and in figures 16 through 19 the Feulgen-positive structures do not show such strands. We think that these fine threads correspond to lampbrush loops, radiating from the axis of the chromosome.

When, with the help of adenosine, the chromatin strands become visible, they often appear as beaded filaments, which resemble chromomeres. This can be studied by motion pictures. In other instances little loops become visible at the border of the nucleolus when it shrinks in size without unfolding. The same phenomena could be observed when hypotonic solutions were used or when actinomycin was applied. These pictures very well fit the descriptions given for the behavior of lampbrush chromosomes when exposed to dilute saline [Gall (21)] or to actinomycin [Izawa *et al.* (22)].

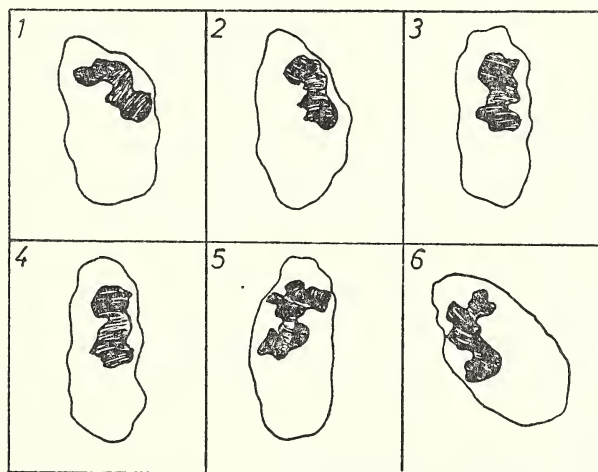
In one of our motion pictures a nucleolus was observed for about 1 hour prior to mitosis. The nucleolus divided in half but, before this happened, loops could be seen on one border of the nucleolus where they extended and retracted (text-fig. 1).

FORMATION OF RIBBON-LIKE NUCLEOLONEMAS

In another part of the motion picture, the accumulation of nucleolar material around the chromatin strands, which had been unmasked before through the action of adenosine, can be seen. Here again we have indications of the existence of loops which interlace between two chromatin strands and become closely connected by the aid of the accumulated nucleolar material. Another part of the motion picture shows a ribbon-like nucleolus (text-fig. 2), which may have originated in the same way. These pictures are comparable to those shown in text-figure 3.

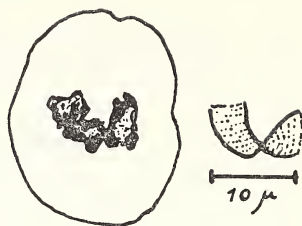


TEXT-FIGURE 1.—Diagram of the nucleolus of a chicken fibroblast taken from a frame-by-frame evaluation of a motion picture. About 30 minutes elapsed between the taking of the first and last pictures. On the *lower border* of the nucleolus tiny little loops became visible and then disappeared.



TEXT-FIGURE 2.—Diagram of the nucleolus of a chicken fibroblast taken from a frame-by-frame evaluation of a motion picture. About 30 minutes elapsed between the taking of the first and last pictures. The nucleolus looks like a coiled ribbon changing its shape in a very remarkable way.

TEXT-FIGURE 3.—Schematic drawing from a Feulgen picture of the nucleolus of a HeLa cell. Two chromatin strands are connected by loops. The drawing beside the nucleus represents a ribbon with a turn.



We think that the ability of chromatin to form ribbon-like structures with protruding loops may be a clue for interpretation of the "lamellar" chromatin structures observed by Granboulan and Granboulan (10).

PROBLEMS OF THE FEULGEN REACTION OF THE INTRANUCLEOLAR CHROMATIN

There is a puzzling discrepancy between our results obtained with the Feulgen reaction and those obtained by most investigators.

The following are some exogenous and endogenous factors important for the Feulgen reaction of the intranucleolar chromatin:

- A) EXOGENOUS FACTORS : a) flattening of the nucleus
 - b) kind of fixation
 - c) duration of hydrolysis
- B) ENDOGENOUS FACTORS: a) amount and consistency of the coacervate-like nucleolar material (more dense or more fluid) ; proportion of RNA, protein, lipids; disaggregation (vacuole formation)
 - b) length, number, and arrangement of chromatin strands and their degree of condensation (lampbrush loops)

As far as we can see, the result of the reaction is closely related to the amount and consistency of the nucleolar material which not only coats the structures but is more often embedding them. There are fixatives which apparently give a precipitate which makes the chromatin structures inaccessible to the Feulgen procedure. This is especially the case with formalin fixation. Only rarely have we obtained a positive reaction of the chromatin strands after formalin fixation, even with well-flattened cells. Fixation according to Serra proved to be rather reliable, but the reaction may be negative when a great amount of dense material covers the strands or when the cells are not sufficiently flattened. The duration of the hydrolysis is not equally optimal for the nucleolar and the rest of the chromatin. Here we refer to the work of Agrell (23). At any rate, paired chromatin strands of the nucleoli of polyploid well-flattened cells are most favorable for a strong Feulgen reaction. This can be obtained in controls and in an even higher percentage of cells in cultures pretreated with adenosine. In general the red color of such cultures is more brilliant than the color of the controls. Counterstaining with light green may completely obliterate the red stain of the filaments. Occasionally a fine meshwork of threads shines through the green stain.

ELECTRON MICROSCOPY WITH DIFFERENT METHODS OF PREPARATION

Our investigations with the electron microscope were intended to coordinate light microscopic observations in living and in stained cells with the electron micrographs of the same material. We were especially interested in the demonstration of different components of the nucleolonema.

We have to keep in mind that the amount of DNA in proportion to RNA, protein, and lipids is not high when the strands are embedded in the nucleolar material; thus a very distinct effect of the action of deoxyribonuclease cannot be expected in the fixed preparations. Although in lampbrush chromosomes the direct proof of the chromatin nature of their axes can be given by the action of deoxyribonuclease which breaks the loops, the same cannot be done in our material. We therefore started investigations, comparing various methods of preparation which had proved successful in demonstrating different components of the nucleolus in the light microscope.

Figures 28 and 29 are pictures of chicken fibroblasts and figures 30 and 31 pictures of HeLa cells.

Figure 28 comes from a culture fixed with formalin, followed by OsO_4 . There is an evenly stained area corresponding to the nucleolus where it is difficult to distinguish between the nucleolonema and nucleolar material.

Figure 29 shows fixation with OsO_4 only. There is an indication of a special affinity of parts of the nucleolonema to OsO_4 .

Figure 30 demonstrates the result of glutaraldehyde fixation followed by silver impregnation according to Gonzalez-Ramirez (17). Here again a specific affinity of some segments of the nucleolonema to the impregnation is clearly visible.

Figure 31 represents fixation with KMnO_4 . Here, a kind of axis of the filamentous part is visible, and a rather well-defined area—corresponding to the center of the nucleolus—appears denser than the rest of the nucleus. The dense area is surrounded by a clearer halo into which strands from the dense center radiate.

We think that this could mean that the site of decondensed chromatin material is here marked by a reaction of its coating surface layer with KMnO_4 . The finely decondensed chromatin strands are more densely packed in the center of the nucleolar area than in the rest of the nucleus.

CONCLUSIONS

At the beginning of the paper, we proposed that according to our studies the nucleolonema has to be regarded as a complex formation composed of different materials, its "backbone" being DNA strands.

Our results eliminate the controversies in regard to the Feulgen reaction of the intranucleolar filaments. We have shown that the result of the Feulgen reaction depends on various factors, such as fixation, consistency,

and amount of the nucleolar material, and degree of flattening of the nucleus (*see* p. 109). *Under proper conditions the nucleolonema gives a positive Feulgen reaction.*

The nature of the so-called nucleolonema can also be demonstrated in prophase, when the chromatin segments can be seen to emerge from the nucleolus (demonstrated in a motion picture). We could show that the chromatin segment, involved in the formation of the nucleolus, can be of considerable length. This does not agree with the common assumption that only the so-called secondary constriction is related to the formation of the nucleolus. Our observations in this respect are in good agreement with those of Resende (24), Godward (25), and Chen (26).

Observations made in both living cells and in fixed and stained cells suggest the existence of loops originating from the intranucleolar chromatin strands. Two strands may become connected by interlacing loops and their adhering material. This leads to ribbon-like formations, perhaps corresponding to the "lamellar" structures observed by Granboulan and Granboulan. It may also lead to horseshoe-shaped formations comparable to those demonstrated by Godward. A Feulgen picture of a ribbon-like chromatin strand of the nucleolus of a HeLa cell (text-fig. 3) suggests the possibility that such formations may be responsible for the appearance of the so-called "nucleolus-associated chromatin." In the center of the nucleolus the loops are present, too finely decondensed to give a positive Feulgen reaction when embedded in dense nucleolar material, whereas the chromosomal strands at the periphery give a positive Feulgen reaction.

All available data favor the assumption that as long as nucleolar RNA, hybridizing with the nucleolar chromatin, is being synthesized, functionally active chromatin is present. This does not exclude the possibility that droplets of nucleolar material may be found in the interphase nucleus, detached from the chromatin strands, nor does it exclude the possibility that RNA from other sources may become incorporated into the nucleolar material.

RESUMEN

Este trabajo trata de observaciones sobre estructuras filamentosas en el nucleolo de fibroblastos y células tumorales en cultivo de tejido en condiciones normales y experimentales. Se ha empleado microscopía óptica y electrónica.

Cuando se fijan ciertas condiciones que dependen de la fijación, grado de aplastamiento de las células y situación metabólica del nucleolo los filamentos dan una reacción Feulgen positiva. Esto los caracteriza como partes intranucleolares de los cromosomas. Su largo, a veces considerable, contradice la suposición de que ellos representan sólo la constricción secundaria de los cromosomas. El desplegado de los nucleolos por ejemplo, mediante adenosina también ayuda a demostrar muy claramente los filamentos. Ellos fueron demostrados con éxito mediante micrografías electrónicas, KMnO_4 , y mediante impregnación argéntica.

Ocasionalmente, sobre los bordes nucleolares pueden observarse bucles que se expanden y se retraen. Puede hallarse en preparaciones teñidas indicación de formaciones semejantes a bucles a partir de filamentos de cromatina intranucleolar.

Dos filamentos de cromatina pueden formar una estructura semejante a una cinta con vueltas que se entrelazan, adhiriéndose entre sí por el material sintetizado. La reacción negativa del Feulgen en la región central pero la tinción de los filamentos periféricos explicaría la aparición de la "cromatina asociada al nucleolo."

Los autores considera al "nucleolonema"—de acuerdo con sus resultados—como una formación compleja, compuesta de diferentes materiales, siendo su "esqueleto" de naturaleza cromosómica. Las observaciones indican que las regiones funcionalmente activas de la cromatina intranucleolar pueden presentarse en estado de cromosomas plumulados.

REFERENCES

- (1) ESTABLE, C., and SOTELO, J. R.: Una nueva estructura celular: el nucleolonema. *Publ Inst Inv Cien Biol* 1: 105-126, 1951.
- (2) LETTRÉ, R., and SIEBS, W.: Zur Struktur des Nucleolus. *Naturwissenschaften* 41: 458, 1954.
- (3) ———: Beobachtungen am Nucleolus in vitro gezüchteter Zellen. *Z Krebsforsch* 60: 19-30, 1954.
- (4) ———: Beobachtungen zur Struktur des Nucleolus in normalen Zellen sowie in Tumorzellen. *Z Krebsforsch* 60: 564-580, 1955.
- (5) LETTRÉ, R.: Observations on the behavior of the nucleolus of cells in vitro. *In Fine Structure of Cells*. Leiden, 8th Congress of Cell Biology, 1954. Groningen, Noordhoff Ltd., 1955, pp 141-150.
- (6) LETTRÉ, R., and SIEBS, W.: Some studies of the nucleolus of cells cultivated in vitro. *Path Biol (Paris)* 9: 819-823, 1961.
- (7) ———: Behavior of nucleoli of cells in vitro. 22d International Congress of Physiological Sciences, Leiden. *Proc Int Union Phys Sci*, 1962, pp 837-840.
- (8) ———: Zur Struktur des Nukleolus (Lichtmikroskopische, elektronenmikroskopische und autoradiographische Methoden). Heidelberg, Symposium der Gesellschaft für Nuclearmedizin, 1964. *In Radionuklide in der klinischen und experimentellen Onkologie* (Hoffman, G., and Scheer, K. G., eds.). Stuttgart, F. K. Schattauer-Verlag, 1965, pp 137-149.
- (9) WEISSENFELS, N.: Struktur und Verhalten der Nukleolen von Hühnerherzmyoblasten in Gewebekultur während des Interphasewachstums und der Mitose. *Z Zellforsch* 62: 667-700, 1964.
- (10) GRANBOULAN, N., and GRANBOULAN, P.: Cytochimie ultrastructurale du nucléole. I. Mise en évidence de chromatine à l'intérieur du nucléole. *Exp Cell Res* 34: 71-87, 1964.
- (11) HAY, E. D., and REVEL, J. P.: The fine structure of the DNP component of the nucleus. An electron microscopic study utilizing autoradiography to localize DNA synthesis. *J Cell Biol* 16: 29-51, 1963.
- (12) MONTY, K. J., LITT, M., KAY, E. R. M., and DOUNCE, A. L.: Isolation and properties of liver cell nucleoli. *J Biophys Biochem Cytol* 2: 127-145, 1956.
- (13) DESJARDINS, R., SMETANA, K., and BUSCH, H.: A large scale procedure for isolation of nucleoli from rat liver. *Exp Cell Res* 40: 127-137, 1965.
- (14) HARRIS, H.: The initiation of deoxyribonucleic acid synthesis in the connective tissue cell, with some observations on the function of the nucleolus. *Biochem J* 72: 54-60, 1959.
- (15) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nuclear organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (16) HUGHES, A.: The effects of purines and related substances upon cells in chick tissue cultures. *Exp Cell Res* 3: 108-120, 1952.
- (17) GONZALEZ-RAMIREZ, J.: Nucleolar physiology. III. Considerations about the meaning of nucleolar silver impregnations in cultured HeLa cells. *Bol Inst Estud Med Biol (Mex)* 19: 195-206, 1961.

- (18) BERENBAUM, M. C.: The histochemistry of bound lipids. *Quart J Micr Sci* 99: 231-242, 1958.
- (19) SUSKIND, R. G.: Autoradiographic and cytochemical evidence for synthesis of a lysine-containing ribonucleoprotein in nucleoli inhibited by actinomycin D. *J Cell Biol* 24: 309-316, 1965.
- (20) VINCENT, W. S.: Structure and chemistry of nucleoli. *Intern Rev Cytol* 4: 269-298, 1955.
- (21) GALL, J. G.: Chromosomal differentiation. *In* The Chemical Basis of Development (McElroy, W. D., and Glass, B., eds.). Baltimore, The Johns Hopkins Press, 1958, pp 103-135.
- (22) IZAWA, M., ALLFREY, V. G., and MIRSKY, A. E.: The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc Nat Acad Sci USA* 49: 544-551, 1963.
- (23) AGRELL, I., and BERGQVIST, H.-A.: Cytochemical evidence for varied DNA complexes in the nuclei of undifferentiated cells. *J Cell Biol* 15: 604-606, 1962.
- (24) RESENDE, F.: Über die Chromosomenstruktur in der Mitose der Wurzelspitzen. II. Sat-Differenzierungen, Spiralbau und Chromonemata. *Chromosoma* 1: 486-520, 1940.
- (25) GODWARD, M. B. E.: On the nucleolus and nucleolar-organizing chromosomes of *Spirogyra*. *Ann Bot* 14: 39-53, 1950.
- (26) CHEN, T.-T.: Chromosomes in Opalinidae (Protozoa, Ciliata) with special reference to their behavior, morphology, individuality, diploidy, haploidy, and association with nucleoli. *J Morph* 83: 281-358, 1948.

PLATE 21

FIGURES 1, 2, AND 3.—Living chicken fibroblast, phase-contrast. Figure 1: untreated; figures 2 and 3: 15 and 55 minutes, respectively, after the addition of adenosine (final concentration: 2 mM). The chromosomal structure of the nucleolus becomes visible. *Note* dark droplets of nucleolar material. (Staining with methyl green-pyronine would give green chromosomal structures and pink droplets.) $\times 1,575$

FIGURE 4.—Electron micrograph of thick section of chicken fibroblast treated with adenosine for 75 minutes, prefixed with formalin and fixed with OsO_4 . *Note* continuity of chromosomal structure of the nucleolus and the droplets of nucleolar material. $\times 23,000$

FIGURES 5, 6, AND 7.—Human tumor cell (HeLa). Figure 5: living cell, untreated, phase-contrast. Figure 6: the same cell, 95 minutes after addition of adenosine (final concentration: 2 mM), phase-contrast. Figure 7: Feulgen preparation of same nucleus (fixed, according to Serra's method, in a mixture of 30 parts absolute alcohol, 15 parts formalin, and 1 part acetic acid). *Note* Feulgen-positive filamentous structures of nucleolus. $\times 1,575$

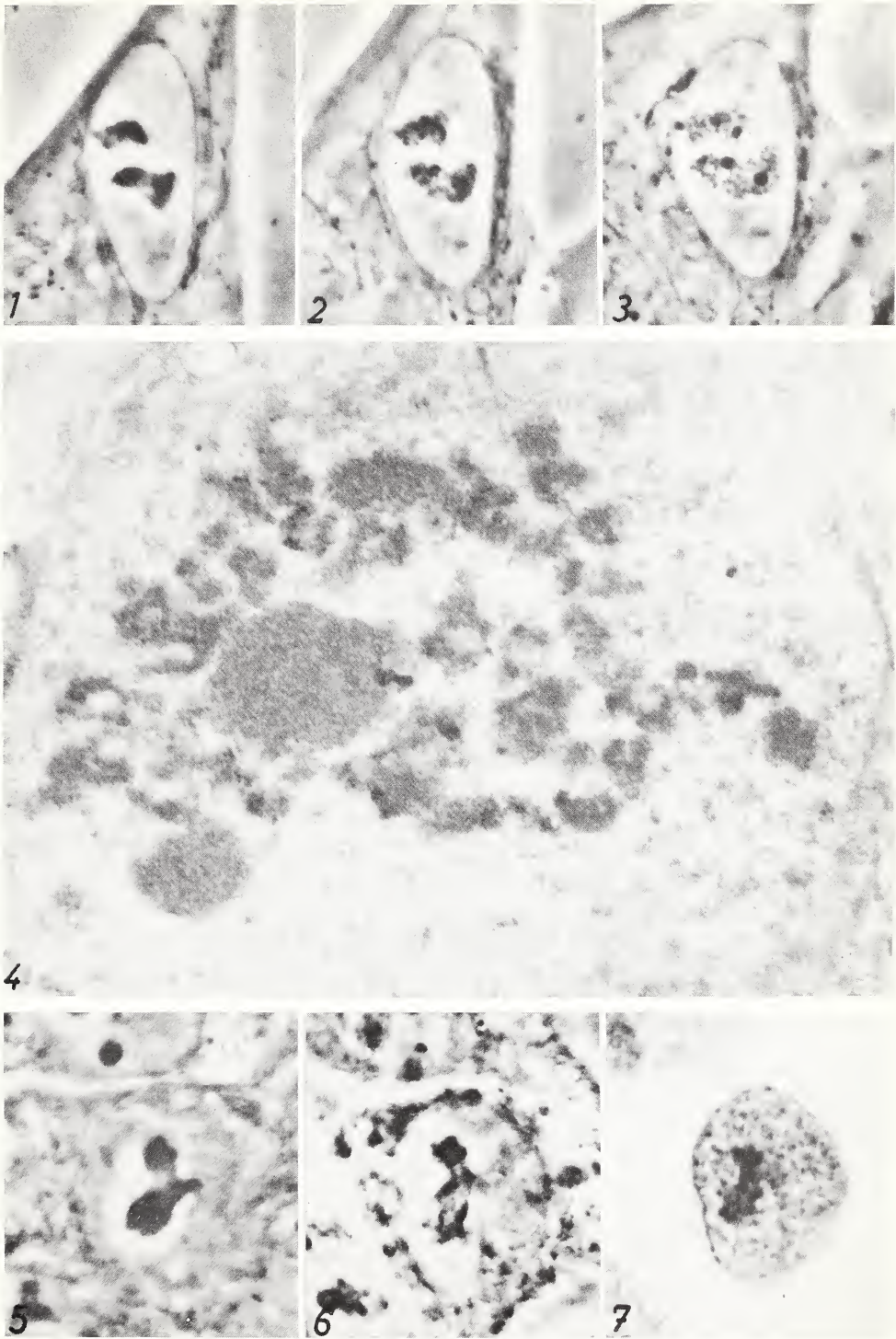


PLATE 22

Figures 8 through 27.—Human tumor cells (HeLa). Figures 8 through 15: $\times 1,700$. Figures 16 through 27: $\times 1,100$

FIGURES 8 AND 12.—Fixation with KMnO_4 . *Note* granular appearance of nucleolus.

FIGURE 9.—Preparation for bound lipids by Berenbaum's technique. The nucleolus is almost uniformly stained.

FIGURE 13.—Same photograph as figure 9, but underprinted.

FIGURES 10, 11, 14, AND 15.—Silver impregnation by method of Gonzalez-Ramirez. The argyrophilic material is arranged like beads on a string. Figure 10: fixation with glutaraldehyde. Figure 14: Same photograph as figure 10, but underprinted. Figure 11: fixation with formalin. Figure 15: same photograph as figure 11, but underprinted.

FIGURES 16 THROUGH 19.—Feulgen reaction (cells fixed by method of Serra in a mixture of 30 parts absolute alcohol, 15 parts formalin, and 1 part acetic acid). *Note* Feulgen-positive structures of the "nucleolonema."

FIGURES 20 THROUGH 27.—Hemalum preparations. Figures 20 through 23: same photographs as figures 24 through 27, but underprinted. *Note* fine strands, radiating at a right angle from the chromosome axis.

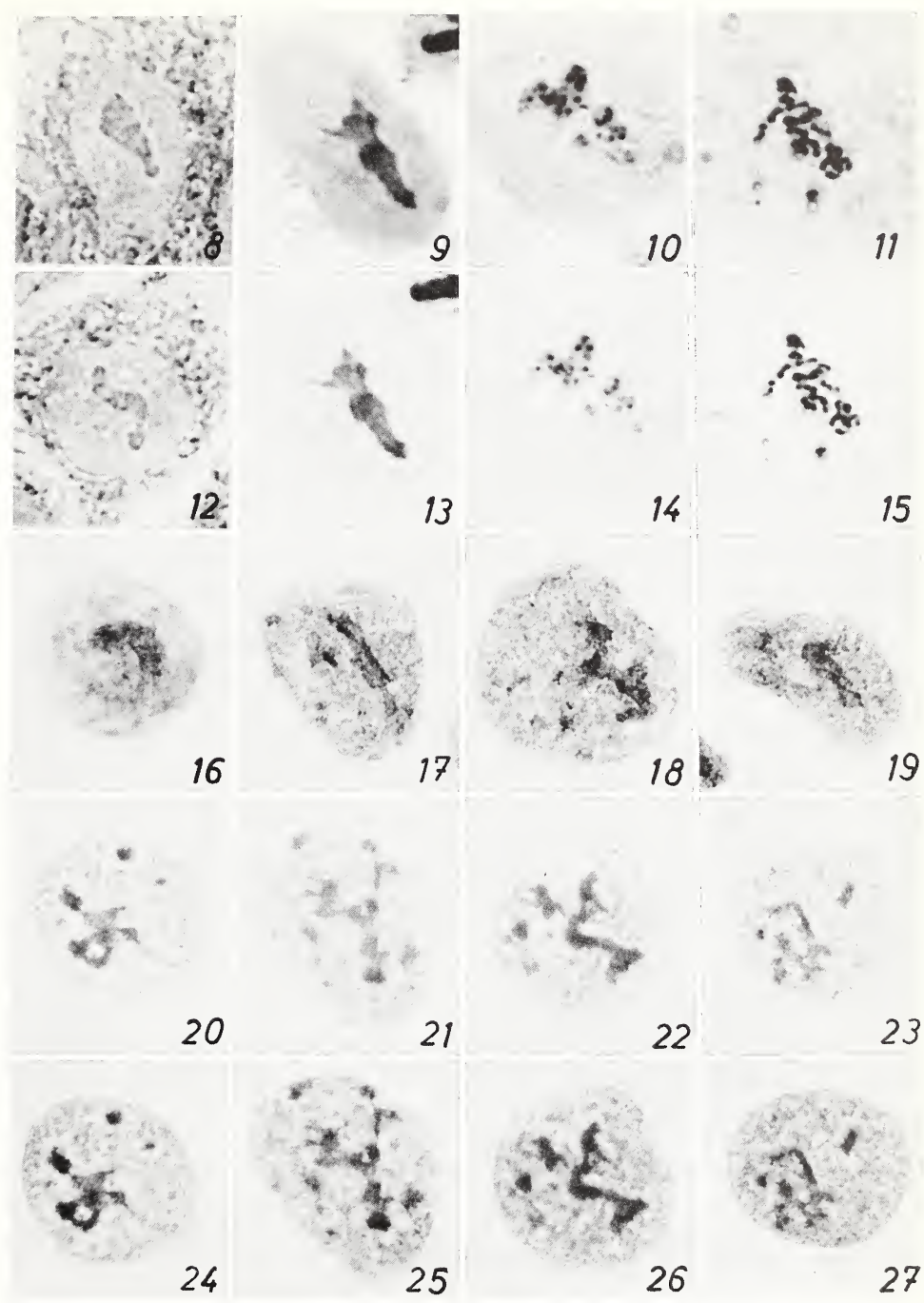


PLATE 23

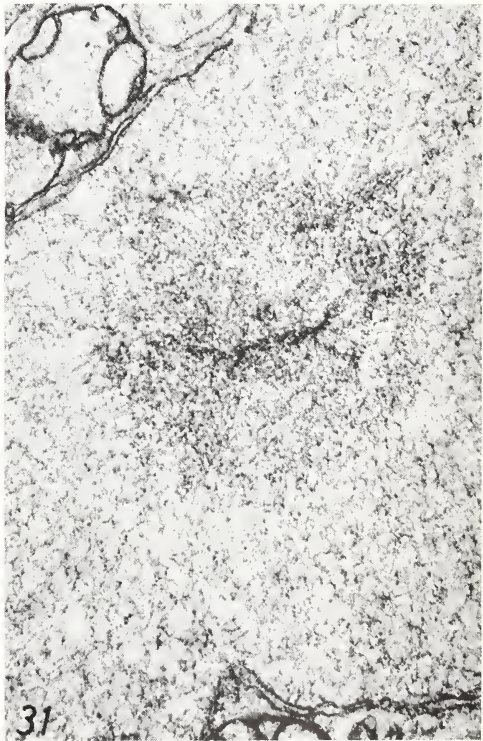
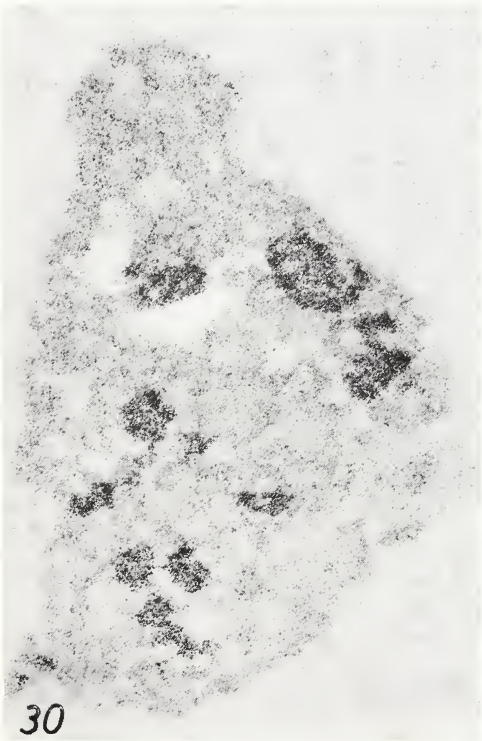
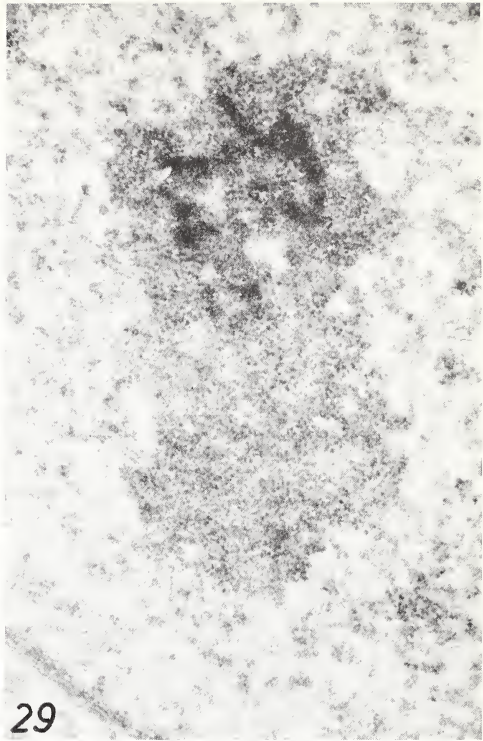
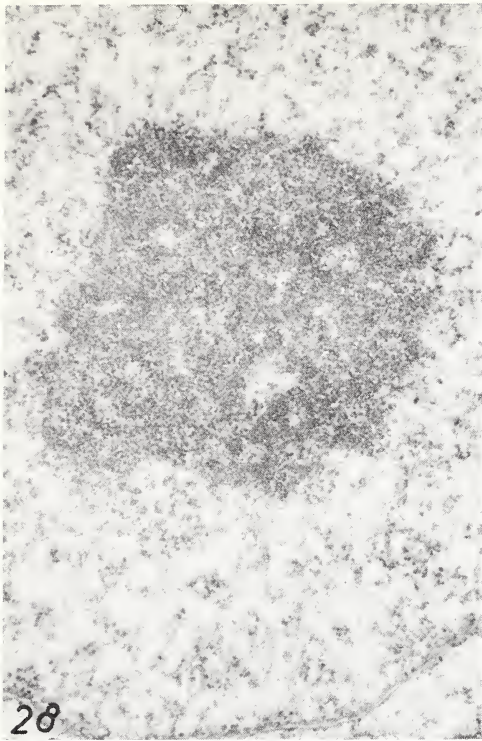
Figures 28 through 31.—Electron micrographs of thin sections. Different methods of preparation. $\times 30,000$

FIGURE 28.—Nucleolus of chicken fibroblast prefixed with formalin and fixed with OsO_4 . A distinction between filamentous structures and nucleolar material is impossible.

FIGURE 29.—Nucleolus of chicken fibroblast fixed with OsO_4 only. Parts of the "nucleolonema" are intensely stained.

FIGURE 30.—Nucleolus of a HeLa cell fixed with glutaraldehyde and impregnated with silver by technique of Gonzalez-Ramirez. Several areas of the nucleolus, which resemble the osmiophilic parts, are strongly impregnated.

FIGURE 31.—Nucleolus of HeLa cell fixed with KMnO_4 . In the darker area of the nucleus, which corresponds to the nucleolus, the nuclear fibrils are more densely packed.



Nucleolar Architecture in Root Meristematic Cells of *Allium cepa*¹

L. A. CHOUINARD, *Anatomy Department, Laval University, Quebec City, Canada*

SUMMARY

A correlated light and electron microscope study of *Allium cepa* revealed that the interphase nucleolus is made up of four components, each segregated into zones distinguishable by differences in staining properties and ultrastructural characteristics. These components are referred to as the pars amorpha, the nucleolonema, the vacuoles enclosed within the pars amorpha, and finally, the vacuoles contained within the nucleolonema. In Feulgen preparations counterstained with methylene blue, the pars amorpha, which usually makes up the bulk of the dense portion of the nucleolar mass, stained more intensely than the nucleolonema which occupies the central and peripheral or bordering regions of the nucleolus as well as narrow spaces extending more or less radially in between. The minute vacuoles confined to the pars amorpha and the larger ones contained within the nucleolonema, however, remained unstained. Under the electron microscope, the pars amorpha appeared to be made up of tightly packed fibrils ranging from 60 to 100 A in diameter

interspersed with electron-opaque granules averaging 140 A in diameter; the vacuoles of the pars amorpha contained loosely arranged fibrils whose diameter varied from 60 to 100 A. The nucleolonema, on the other hand, consisted of densely packed ribosome-like 150 A granules and convoluted fibrils ranging from 100 to 150 A in diameter. The vacuoles of the nucleolonema contained loosely and uniformly scattered fibrils and granules approximately 150 A in diameter. During prophase, the nucleolar components described above gradually disappeared from view in the following order: the vacuoles of the nucleolonema, the nucleolonema, the vacuoles of the pars amorpha, and, finally, the remnants of the pars amorpha. During nucleolar reconstitution at telophase, the same components gradually reappeared in the following sequence: pars amorpha, the vacuoles of the pars amorpha, the nucleolonema and, finally, at post telophase, the vacuoles of the nucleolonema.—*Nat Cancer Inst Monogr* 23: 125-143, 1967.

THE CURRENT CONCEPT of nucleolar architecture, based mostly on electron microscopic studies in animal cells, recognizes at least two fundamental intranucleolar components, an apparently structureless pars amorpha and a threadlike element, the nucleolonema, usually disposed as

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5-10, 1965.

to that of the two ultrastructurally distinct components present in *A. cepa* nucleoli. In a number of animal nucleoli, indeed, the central region of the nucleolus is usually occupied by pars amorpha whereas the peripheral region of the nucleolar mass consists predominantly of nucleolonematic strands (16-20). In *A. cepa* also, the areas of predominantly fibrillar material, allegedly corresponding to pars amorpha, were always surrounded by a fibrillogranular material exhibiting the characteristics of nucleolonema.

In addition to what will be referred to as nucleolonema and pars amorpha, our observations revealed the existence within the nucleolar mass in *A. cepa* of a number of vacuoles of varying size and shape which remained unstained following the Feulgen-methylene blue procedure; quite often one of these vacuoles was located more or less centrally and occupied a relatively large portion of the nucleolar mass (figs. 1 through 4). A sizable number of nucleoli, however, contained only small, often barely recognizable, vacuoles. Mainly on the basis of their topographical distribution and ultrastructural characteristics, two types of vacuoles could be recognized within the nucleolus in the species investigated. The vacuoles of the first type were usually large or medium size, contained loosely and uniformly scattered fibrils and granules averaging 150 A in diameter, and were observed exclusively within the nucleolonematic zones of the nucleolus. The vacuoles of the second type were always small, contained loosely arranged convoluted fibrils 60 to 100 A in diameter, and were confined to the pars amorpha of the nucleolus. Intranucleolar vacuoles in both plant and animal cells have usually been thought to form as a result of the physiological activity of the nucleolar matrix (21-25). Assuming that the occurrence of intranucleolar vacuoles in *A. cepa* is also a morphological sign of anabolic activities, there can be little doubt that both the nucleolonema and pars amorpha are sites of active synthesis and accumulation of materials. It is of interest that the content of the larger intranucleolar vacuoles in *Vicia faba* has previously been shown to consist partly of ribonucleoproteins (26). But with respect to the problem of the nature of the smaller vacuoles confined to the pars amorpha of the nucleolus, another possibility must be envisaged. In recent years, indeed, evidence based on electron microscopic cytochemistry as well as autoradiography indicates the presence at the ultrastructural level of DNA or chromatin fibrils within the nucleolus (14, 17, 27-31). In addition, Karasaki (16) has recently shown that the presence of such submicroscopic chromatin fibrils is restricted to the fibrillar zones (pars amorpha according to our interpretation) of the nucleolus in embryonic cells of *Triturus*. In light of the above findings, it might not be too farfetched to postulate that the loosely dispersed 60 to 100 A fibrils, contained within the small vacuoles of the pars amorpha in *A. cepa* nucleoli, represent, in fact, chromatin material of the nucleolar organizer rather than products elaborated by the pars amorpha. Morphological evidence suggesting the presence of chromatin threads, resembling in appearance the nucleolar organizing chromosomal

material inside nucleolar vacuoles (lacunae, interstices) has already been presented by Maggio *et al.* (32) in the case of mammalian liver cells, by Falk (33) in *A. cepa* root meristematic cells, and, finally, by Godward and Jordan (34) in *Spirogyra*.

The Prophase and Telophase Nucleolus

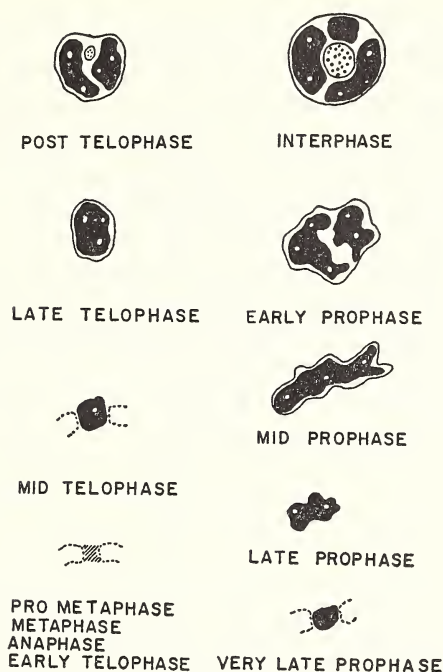
During early prophase, the nucleolus became more and more irregular in contours (fig. 6) and, by midprophase, large, pointed nucleolar projections extended between neighboring chromosomes (fig. 7). During late prophase, the irregularly shaped nucleolar mass decreased rapidly in size (fig. 8) and, by very late prophase, the only remnant of the disintegrating nucleolus was represented by a small, rounded body slightly less intensely stained than the chromosomes (fig. 9); such a nucleolar remnant appears to be intimately associated with a chromosome segment, very likely the nucleolar organizing secondary constriction of the nucleolar chromosome in the species investigated. Just before breakdown of the nuclear envelope, all remnants of the nucleolus as a formed body had disappeared. As in the case of the interphase nucleolus, the prophase nucleolus, at least up to about the middle of late prophase, was seen under both the light and electron microscopes to be made up of two main components segregated into distinct zones, namely, nucleolonema and pars amorpha (figs. 6, 7, and 8). The large or medium size vacuoles contained within the nucleolonematic portion of the interphase nucleolus gradually disappeared during very early prophase; the smaller vacuoles confined to the pars amorpha, on the other hand, apparently persisted until about the middle of late prophase. From midprophase to late prophase and onward, what was left of the disintegrating nucleolar mass was made up exclusively of remnants of the pars amorpha and this also applied to the small nucleolar body persisting within the nuclear cavity up to very late prophase. The final steps in the dissolution of the remnants of the pars amorpha just before breakdown of the nuclear membrane have not been actually observed in the electron microscope. With regard to this problem, it is conceivable that the low density fibrillar material that fills the nucleolar secondary constrictions from prometaphase to midtelophase in *V. faba* (?) represents, in fact, what is finally left of the pars amorpha remnant following completion of nucleolar disintegration. It is of interest that such a material has previously been interpreted as indicating formation of precocious nucleolar material (35, 36).

The early telophase nucleus was characterized by the appearance and accumulation within the interchromosomal spaces of a material which stained cabbage green following the Feulgen-methylene blue procedure (fig. 10). Under the electron microscope, this material was seen to consist of loosely arranged fibrillar elements, 60 to 100 Å in diameter, intermingled with dense 150 Å granules indistinguishable from those observed in the surrounding cytoplasm. As the nucleus entered midtelophase, the

fibrillogranular interchromosomal material just described disappeared rapidly and this phenomenon was concomitant with the appearance of the youngest telophase nucleoli seen or recognized as a formed body in the present study (fig. 11). At that early stage of its development (fig. 14), the rounded nucleolus was seen, under the electron microscope, to be made up mainly, if not exclusively, of densely packed convoluted fibrils 60 to 100 Å in diameter; the few granule-like structures observed within the nucleolar mass were, indeed, probably best interpreted as cross sections of the larger constituent fibrils. One or two small vacuole-like structures, the content of which exhibited a rather loose fibrillar texture, were also present within these young nucleoli. It is of interest that the earliest recognizable telophase nucleoli in *A. cepa* already showed ultrastructural characteristics that bore a strong resemblance to those of the pars amorpha in the interphase nucleoli; the electron-opaque granules, characteristic of the pars amorpha in the mature nucleolus were, however, not yet observable in these early forming nucleoli. As telophase progressed, the nucleolus continued to increase in size (fig. 12). Such nucleolar growth is brought about partly by an increase in the amount of fibrillar material (pars amorpha) and partly by the acquisition in the peripheral portion of the nucleolar mass of a fibrillogranular material, consisting of both ribosome-like 150 Å granules and convoluted fibrils ranging from 100 to 150 Å in diameter (fig. 15); judged from its ultrastructural characteristics, this newly acquired nucleolar component can be equated with the nucleolonema seen in the interphase nucleolus. By very late telophase or early post telophase (fig. 13), the nucleolus was seen, under both the light and electron microscopes, to exhibit the main organizational features of the more mature nucleolus, namely, a compact fibrillar pars amorpha interspersed with uniformly scattered electron-opaque granules, a dense fibrillogranular nucleolonema and, finally, vacuoles confined to the pars amorpha and nucleolonema.

Text-figure 1 illustrates schematically our views of the behavior of the four nucleolar components during mitosis in the species investigated. In the course of prophase, these components gradually disappeared in the following order: the vacuoles of the nucleolonema, the nucleolonema, the vacuoles of the pars amorpha, and, finally, the remnants of the pars amorpha. During nucleolar reconstitution at telophase, the same components gradually reappeared in the following sequence: pars amorpha, the vacuoles of the pars amorpha, the nucleolonema, and, finally, at post telophase, the vacuoles of the nucleolonema.

Although it is well established that telophase nucleoli form in specific sites on the so-called nucleolar chromosomes (37, 38), it is not yet known whether such sites merely serve to collect nucleolar material dispersed elsewhere in the nucleus or whether they actually synthesize the nucleolar material. A number of workers have presented data suggesting that the nucleolar material first appears in the form of a coating of droplets on the surface of the telophase chromosomes and is subsequently simply collected



TEXT-FIGURE 1.—Diagram illustrating the behavior of the four structural components of *Allium cepa* nucleolus (nucleolonema and pars amorpha and their enclosed vacuoles) during nucleolar disintegration at prophase and nucleolar reconstitution at telophase. *Solid black areas*: the more intensely stained nucleolar component corresponding to the pars amorpha; *clear areas*: more lightly stained nucleolar component corresponding to the nucleolonema; *rounded stippled areas (small dots)*: vacuoles enclosed within the nucleolonema; *tiny clear spaces within the solid black areas*: vacuoles confined to the pars amorpha. *Thin parallel oblique lines* filling the nucleolar organizing secondary constriction of the nucleolar chromosomes from prometaphase to early anaphase represent a low density fibrillar material, believed to be the final remnant of the pars amorpha following completion of nucleolar disintegration.

at the nucleolar sites [literature citations in (7 and 39)] to form the mature nucleolus.

In the present study, evidence was obtained indicating that growth of the telophase nucleolus, at least from the time it is first recognizable as a formed body, results mainly, if not exclusively, from the synthetic activity of the nucleolar organizer and not from an incorporation of the material that accumulates in the interchromosomal spaces during early telophase. The relevant observed evidence in favor of such a conclusion can be summarized as follows: (a) the young telophase nucleoli exhibited a dense, exclusively fibrillar texture while the interchromosomal material was seen to contain a sizable amount of ribosome-like 150 Å granules (fig. 14); (b) the interchromosomal material largely disappeared from view when the forming nucleolus was still relatively small (figs. 11 and 14); (c) throughout

its early growth period, the surface of the nucleolus was never seen to be continuous with patches of this interchromosomal material (figs. 11 and 14); (d) when the nucleolus underwent rapid growth during late telophase, the interchromosomal material was no longer recognizable as such within the nuclear cavity (figs. 12 and 15). The above observations and conclusions are in agreement with Swift's claim that the material filling the interchromosomal spaces at early telophase has no connection whatsoever with the formation of the telophase nucleolus (40).

The present findings concerning nucleologenesis in *A. cepa* closely parallel those recently reported by Karasaki (16) in *Triturus* embryos. According to this author, the youngest nucleoli (primary nucleoli) originate as small, dense, exclusively fibrous bodies within the chromosomal material. These nucleoli enlarge during successive developmental stages by the acquisition of a component made up predominantly of ribosome-like 150 A granules that form a layer around them. Similarly, in *A. cepa*, the youngest nucleoli seen or recognized at telophase showed a dense fibrous structure (*pars amorpha*) only. Subsequent growth of the nucleolus is brought about partly by the addition in the peripheral portion of the nucleolar mass of a component (nucleolonema) characterized mainly by the presence of 150 A granules resembling the cytoplasmic ribosomes.

The sequential appearance of the *pars amorpha* and nucleolonema during nucleologenesis in *A. cepa* suggests that the fibrillar components of the *pars amorpha* are involved in the formation of both the fibrils and ribosome-like granules present in the nucleolonema. Such a suggestion is in agreement with the autoradiographic data showing a sequential labeling of RNA of fibrous (*pars amorpha*) and granular (nucleolonema) regions in the animal nucleolus, thus indicating that the RNA fibers of the *pars amorpha* represent precursors of the ribosome-like granules formed in the nucleolonema (16, 41, 42). The close association of the chromatin of the nucleolar organizer with the fibrous regions (*pars amorpha*) of the animal nucleolus reported by several authors (16, 20, 43) makes it very likely, however, that this nucleolus-associated chromatin is the ultimate site of nucleolar RNA synthesis. In this connection, it is of particular interest that the small vacuoles, allegedly containing chromatin material, have been shown to be confined to the *pars amorpha* in *A. cepa* nucleoli.

RESUMEN

El estudio correlacionado con el microscopio óptico y el electrónico revela que el nucleolo interfásico, está formado por cuatro componentes, segregados cada uno en zonas distinguibles por diferencias en propiedades tintoriales y en características ultraestructurales. Se refieren estos componentes como la *pars amorpha*, el nucleolonema, las vacuolas incluídas dentro de la *pars amorpha*, y, finalmente, las vacuolas contenidas dentro del nucleolonema. En las preparaciones con Feulgen, contrastadas con azul de metileno, se ve que la *pars amorpha*, que habitualmente compone la mayor parte de la porción densa de la masa nucleolar, se tiñe más intensamente que el nucleolonema, que ocupa las regiones central y periférica o marginal del nucleolo,

como así también los estrechos espacios que se extienden entre él, más o menos radialmente, las pequeñas vacuolas confinadas a la *pars amorphia* y las más grandes contenidas dentro del nucleolonema, permanecen, sin embargo, sin teñirse. Con microscopía electrónica la *pars amorphia* parece estar compuesta de fibrillas estrechamente unidas con diámetros que van de 60 a 100 Å entremezcladas con gránulos opacos a los electrones de 140 Å de diámetro promedio; las vacuolas de la *pars amorphia* contienen fibrillas dispuestas en forma laxa cuyo diámetro varía de 60 a 100 Å. Se ve por otra parte, que el nucleolonema consiste en gránulos densamente unidos semejantes a ribosomas de 150 Å y fibrillas enroscadas que van de 100 a 150 Å en diámetro. Las vacuolas del nucleolonema contienen fibrillas dispersas en forma laxa y uniforme y gránulos de aproximadamente 150 Å de diámetro. En el curso de la profase, los componentes nucleolares descritos anteriormente, desaparecen en forma gradual de la vista y en el siguiente orden: las vacuolas del nucleolonema, el nucleolonema, las vacuolas de la *pars amorphia* y finalmente, los remanentes de la *pars amorphia*. Durante la reconstitución nucleolar en la telofase, reaparecen gradualmente los mismos componentes de acuerdo a la siguiente secuencia: la *pars amorphia*, las vacuolas de la *pars amorphia*, el nucleolonema y finalmente, en la post-telofase, las vacuolas del nucleolonema.

REFERENCES

- (1) FAWCETT, D. W.: The Cell, Its Organelles and Inclusions. Philadelphia, W. B. Saunders Co., 1966.
- (2) SERRA, J. A.: Interpretation of nucleolar inclusion. *Nature* (London) 181: 1544-1545, 1958.
- (3) PORTER, K. R.: Problems in the study of nuclear fine structure. In *Proceedings of 4th Internat Kongr Elektronenmikroskopie* (Bargmann *et al.*, eds.). Berlin, Springer, 1958, vol 2, pp 186-199.
- (4) LAFONTAINE, J. G.: Structure and mode of formation of the nucleolus in meristematic cells of *Vicia faba* and *Allium cepa*. *J Biophys Biochem Cytol* 4: 777-784, 1958.
- (5) RODKIEWICZ, B.: The nucleolus structure in some plant cells. *Exp Cell Res* 18: 407-410, 1959.
- (6) FABERI, F.: Ricerche al microscopio ottico ed elettronico sulla struttura del nucleolo di cellule meristematiche di alcune piante e considerazioni critiche sulla teoria del "nucleolonema." *Caryologia* 16: 715-749, 1963.
- (7) LAFONTAINE, J. G., and CHOUINARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167-201, 1963.
- (8) HYDE, B. B., SANKARANARAYANAN, R., and BIRNSTIEL, M. L.: Observations on fine structure in pea nucleoli in situ and isolated. *J Ultrastruct Res* 12: 652-667, 1965.
- (9) LUFT, J. H.: Improvements in epoxy resin embedding methods. *J Biophys Biochem Cytol* 9: 409-414, 1961.
- (10) DENUES, A. R. T., and MOTTRAM, F. C.: Note on nucleolonemata in human cultured cells. *J Biophys Biochem Cytol* 1: 185-186, 1955.
- (11) BORYSKO, E., and BANG, F. B.: Structure of the nucleolus as revealed by the electron microscope. A preliminary report. *Bull Hopkins Hosp* 89: 468-473, 1951.
- (12) BERNHARD, W., HAGUENAU, F., and OBERLING, C.: L'ultrastructure du nucléole de quelques cellules animales, révélée par le microscope électronique. *Experientia* 8: 58-59, 1952.
- (13) BERNHARD, W., BAUER, A., GROPP, A., and HAGUENAU, F.: L'ultrastructure du nucléole de cellules normales et cancéreuses. *Exp Cell Res* 9: 88-100, 1955.

- (15) BERNHARD, W., and GRANBOULAN, N.: The fine structure of the cancer cell nucleus. *Exp Cell Res Suppl* 9: 19-53, 1963.
- (15) YASUSUMI, G., SAWODA, T., SUGIHARA, R., KIRIYAMA, M., and SUGIOKA, M.: Electron microscope researches on the ultrastructure of nucleoli in animal tissues. *Z Zellforsch* 48: 10-23, 1958.
- (16) KARASAKI, S.: Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J Cell Biol* 26: 937-958, 1965.
- (17) SWIFT, H.: Cytochemical studies on nuclear fine structure. *Exp Cell Res Suppl* 9: 54-67, 1963.
- (18) ———: Nucleic acids and cell morphology in dipteran salivary glands. In *The Molecular Control of Cellular Activity* (Allen, S. M., ed.). New York, McGraw Hill Book Co., 1962, pp. 73-125.
- (19) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. IV. The nucleus of *Smittia parthenogenetica* (Chironomidae) with special reference to the nucleolus and the effects of actinomycin thereon. *J Ultrastruct Res* 11: 315-328, 1964.
- (20) KALNINS, V. I., STICH, H. F., and BENCOSME, S. A.: Fine structure of nucleoli and RNA-containing chromosome regions of salivary gland chromosomes of chironomids and their inter-relationship. *Canad J Zool* 42: 1147-1155, 1964.
- (21) DERMEN, H.: Origin and behaviour of the nucleolus in plants. *J Arnold Arboretum* 14: 282-323, 1933.
- (22) KUSTER, E.: *Die Pflanzenzelle*. Leipzig, Gustav Fisher Verlag, Jena, Germany, 2d ed., 1951.
- (23) PETERS, K.: Variationstatistische Untersuchungen über das Auftreten von Vacuolen in den Nukleolen von Kuhnerherz fibroblasten in Vitro nach der Einwirkung von Röntgenstrahl encephalographen und Kalte. *Z Zellforsch* 44: 14-26, 1956.
- (24) VON STICH, H.: Bau und Funktion der Nukleolen. *Experientia* 12: 7-14, 1956.
- (25) SOUDEK, D.: The correlation between the growth of the cell and nucleolar secretion in *Basidiobolus ranarum*, Eidam. *Exp Cell Res* 20: 447-452, 1960.
- (26) CHOUINARD, L. A.: Evidence for the existence of two types of nucleolar ribonucleoprotein in root meristematic cells of *Vicia faba*. *Canad J Bot* 42: 779-785, 1964.
- (27) ALTMANN, H. W., STOCKER, E., and THOENES, W.: Über chromatin und DNS-synthese in Nucleolus. Elektronen-mikroskopische, autoradiographische und licht mikroskopische untersuchungen am leberzellen von raten. *Z Zellforsch* 59: 116-133, 1963.
- (28) GRANBOULAN, N., and GRANBOULAN, P.: Cytochimie ultrastructurale du nucléole. I. Mise en évidence de chromatine à l'intérieur du nucléole. *Exp Cell Res* 34: 71-87, 1964.
- (29) LEDUC, E., MARINOZZI, V., and BERNHARD, W.: The use of water soluble glycol methacrylate in ultrastructural cytochemistry. *J Roy Microscop Soc* 81: 119-130, 1963.
- (30) MARINOZZI, V.: The role of fixation in electron staining. *J Roy Microscop Soc* 81: 141-152, 1963.
- (31) HAY, E. E., and REVEL, J. P.: The fine structure of the DNP component of the nucleus. An electron microscopic study utilizing autoradiography to localize DNA synthesis. *J Cell Biol* 16: 29-51, 1963.
- (32) MAGGIO, R., SIEKEVITZ, P., and PALADE, G. E.: Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic sub-fractions. *J Cell Biol* 18: 293-312, 1963.
- (33) FALK, H.: Zum Nucleolus-Feinbau bei *Allium cepa*. *Protoplasma* 54: 432-439, 1962.
- (34) GODWARD, M. B. E., and JORDAN, E. G.: Electron microscopy of the nucleolus of *Spirogyra britannica* and *Spirogyra ellipsospora*. *J Roy Micr Soc* 84: 347-360, 1965.

- (35) ALBUQUERQUE, R. M., and SERRA, J. A.: Nucleolar zones of the chromosomes. *Port Acta Biol Ser A* 3: 187-194, 1951.
- (36) RATTENBURY, J. A., and SERRA, J. A.: Types of nucleolus reconstitution in telophase and the question of the "nucleolar organizer." *Port Acta Biol Ser A* 3: 239-245, 1952.
- (37) HEITZ, E.: Die Ursache der gesetzmässigen Zahl, Lage, Form und Grosse pflanzlicher Nukleolen. *Planta* 12: 775-844, 1931.
- (38) McCLINTOCK, B.: The relation of a particular chromosomal element to the development of nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
- (39) STEVENS, B. J.: The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J Cell Biol* 24: 349-368, 1965.
- (40) SWIFT, H.: Studies on nucleolar formation. *In* A Symposium on Molecular Biology. Chicago, Univ Chicago Press, 1959, pp 266-303.
- (41) GRANBOULAN, N., and GRANBOULAN, P.: Etude des sites nucléaires de la synthèse du RNA en autoradiographie à haute résolution après marquage à court terme. *J Microscopie* 3: 37-38, 1964.
- (42) ———: Cytochimie ultrastructurale du nucléole. II. Etude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp Cell Res* 38: 604-619, 1965.
- (43) STEVENS, B. J.: The effect of actinomycin D on nucleolar and nuclear fine structure in the salivary gland cell of *Chironomus thummi*. *J. Ultrastruct Res* 11: 329-353, 1964.

PLATES

PLATE 24

FIGURES 1, 2, 3, and 4.—Light micrographs of interphase nuclei showing that the stainable portion of the nucleolar mass is made up of two components, each segregated into distinct zones distinguishable by slight differences in staining intensity. The more lightly stained component (nucleolonema) is usually seen in the central and bordering regions of the nucleolus as well as in areas of varying width extending in between. The more intensely stained component (pars amorpha) is observed in areas of varying length and width usually occupying the intermediate regions of the nucleolar mass. Two topographically distinct types of unstained vacuoles are also recognizable within the nucleolus. The vacuoles of the first type are large or medium size and are enclosed within the nucleolonematic portion of the nucleolar mass; the vacuoles of the second type are always small, often barely recognizable under the light microscope, and are confined to the pars amorpha of the nucleolus. Diagrams *in insets* facilitate the interpretation of the corresponding light micrographs: *Solid black areas*: the more intensely stained nucleolar component corresponding to the pars amorpha; *clear areas*: the lightly stained nucleolar component corresponding to the nucleolonema; rounded *stippled (small dots) areas*: vacuoles enclosed within the nucleolonema; tiny *clear spaces* within *solid black areas*: vacuoles confined to the pars amorpha. $\times 3,200$

FIGURE 5.—Electron micrograph of an interphase nucleolus. Two types of structural components, one fibrillogranular, the other predominantly fibrillar, each segregated into distinct zones are found within the denser portion of the nucleolus. The fibrillogranular component corresponding to the nucleolonema is observed in the bordering region of the nucleolus and that of the centrally located vacuole as well as in the three areas of different width extending more or less radially in between; in places and more particularly on the fluffy surface of the nucleolus as well as that of the large, centrally located vacuole (V), the constituent fibrils and granules of the nucleolonema appear to be assembled into coarse threadlike structures (t), some 0.1μ in diameter. The predominantly fibrillar component corresponding to the pars amorpha is observed in three elongated areas of varying width, each circumscribed by a *solid black line*, and located in the intermediate portion of the nucleolar mass. In addition to uniformly scattered electron-opaque granules, averaging 140 \AA in diameter, the pars amorpha contains a number of small vacuole-like structures (v). The large central vacuole contains loosely and uniformly scattered fibrils and granules similar to those found within the nucleolonematic portion of the nucleolus; the content of the small vacuoles appears to be essentially fibrillar in texture. $\times 24,000$

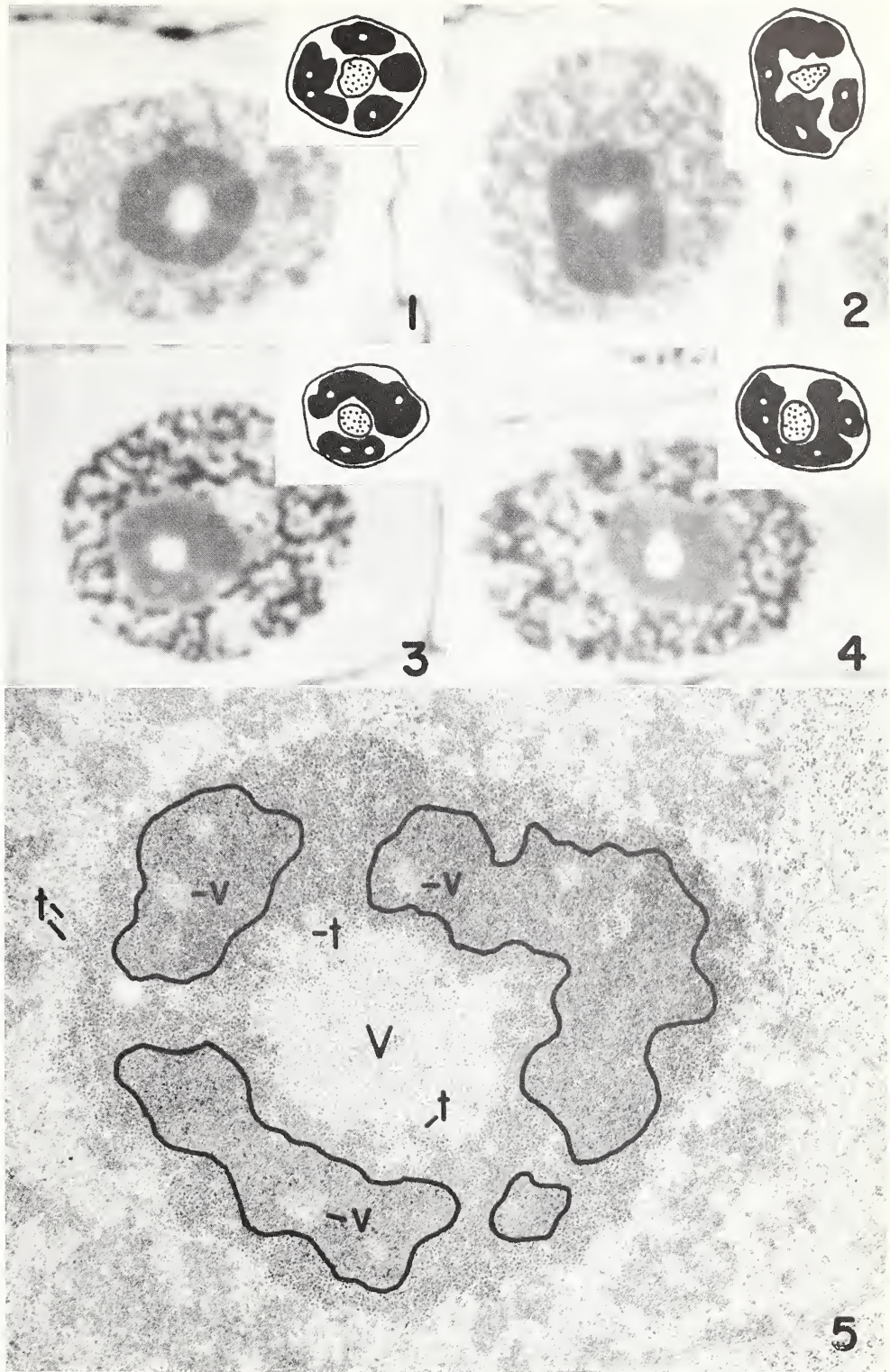


PLATE 25

Figures 6 through 13.—Light micrographs depicting the behavior of nucleolonema and pars amorpha during nucleolar disintegration at prophase (figs. 6 through 9) and nucleolar reconstitution at telophase (figs. 10 through 13). Diagrammatic representations are the same as in figures 1 through 4. $\times 3,200$

FIGURE 6.—Early prophase nucleus. Nucleolonema, pars amorpha, and a large unstained centrally located vacuole are clearly recognizable within the nucleolar mass.

FIGURE 7.—Midprophase nucleus. The irregularly shaped nucleolar mass is made up largely of pars amorpha surrounded by a thin layer of nucleolonema. Four small unstained vacuole-like structures are confined to the pars amorpha.

FIGURE 8.—Late prophase nucleus. The nucleolar mass is reduced in size and now consists almost exclusively of pars amorpha still containing a few enclosed vacuoles.

FIGURE 9.—Very late prophase. The pars amorpha remnant of the disintegrating nucleolus is represented by a small body (*arrow*) slightly less intensely stained than the chromosomes. This body appears to be associated with two chromosome segments, possibly belonging to the nucleolar chromosomes.

FIGURE 10.—Early telophase nucleus. A material (*arrows*) slightly less intensely stained than the chromosomes fills the interchromosomal spaces.

FIGURE 11.—Young midtelophase nucleus at about the time when the nucleolus (*n*) is first recognized as a formed body. At that stage, the forming nucleolus is made up exclusively of pars amorpha containing one or two barely visible vacuoles. Most of the interchromosomal material has disappeared from view.

FIGURE 12.—Very late telophase nucleus. The nucleolus has increased in size and consists of both pars amorpha with enclosed vacuoles and a thin peripheral layer of nucleolonema.

FIGURE 13.—Early post-telophase nucleus. The stainable portion of the nucleolar mass is made up of two components (nucleolonema and pars amorpha), each segregated into distinct zones. Small vacuoles have also begun to form in the nucleolonematic regions of the nucleolus.

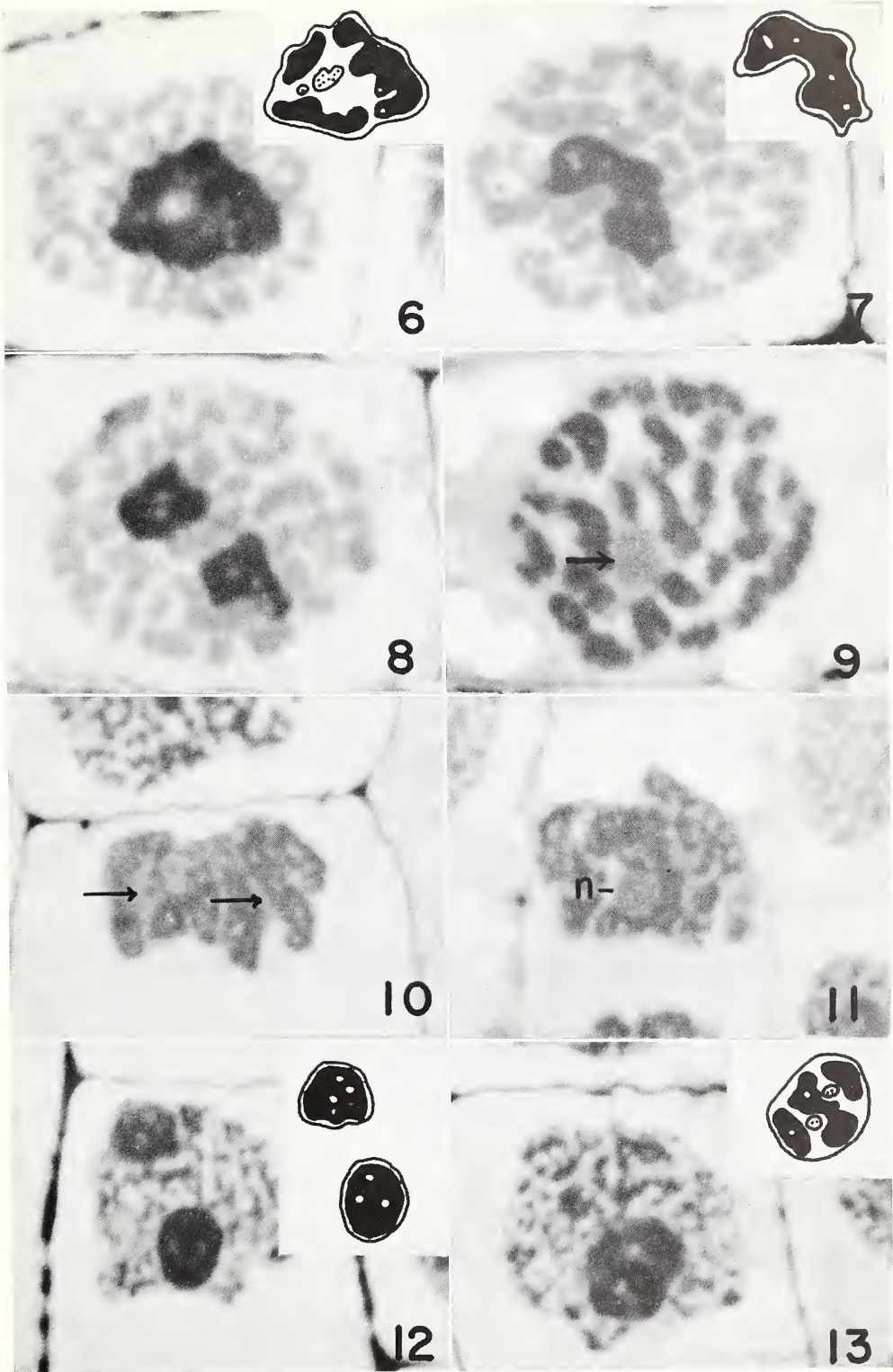
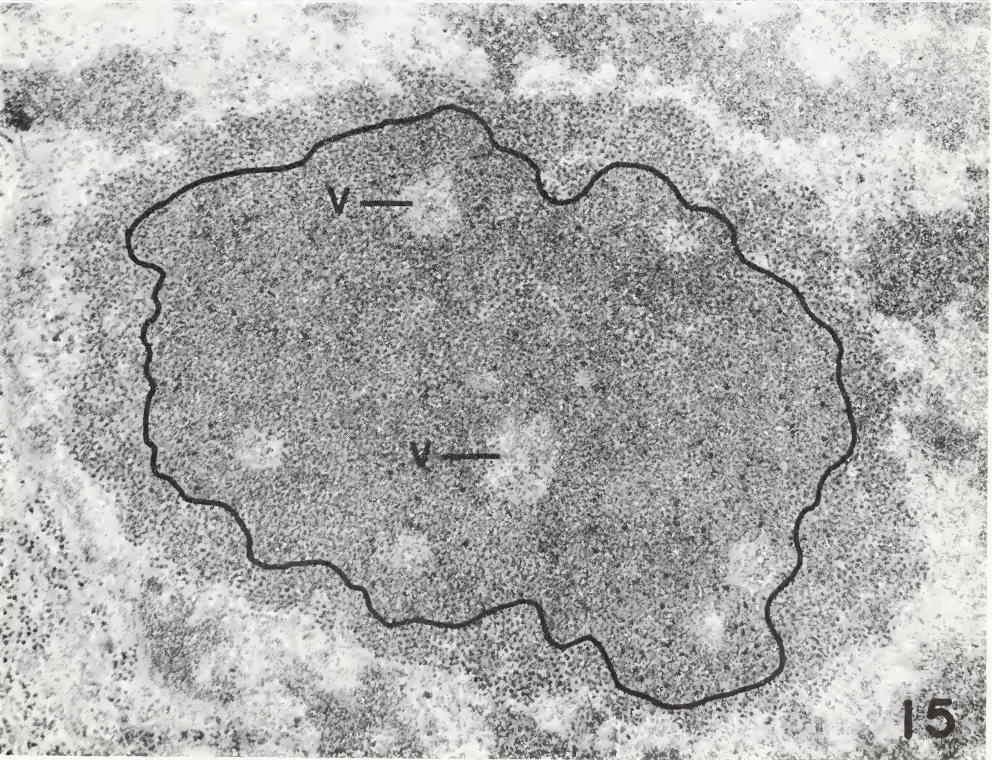
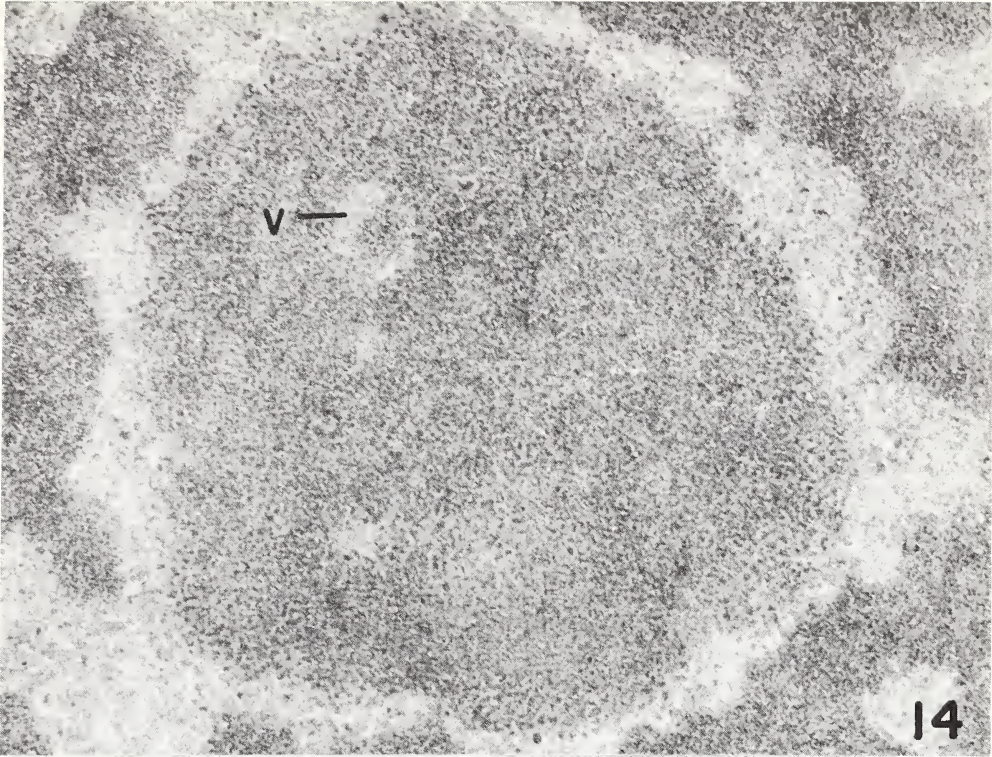


PLATE 26

FIGURE 14.—Young midtelophase nucleolus. The nucleolar mass is made up mainly, if not exclusively, of tightly packed convoluted fibrils 60 to 100 Å in diameter apparently similar to those found within the pars amorpha of a mature nucleolus; one vacuole-like structure (*v*) is enclosed within the fibrillar nucleolar mass. $\times 40,000$

FIGURE 15.—Late telophase nucleolus. Bulk of dense portion of nucleolus consists of pars amorpha (circumscribed by *solid black line*) containing a number of vacuole-like structures (*v*). Nucleolonema forms a layer of variable thickness over the pars amorpha. Ultrastructural characteristics of these two nucleolar components are similar to those of pars amorpha and nucleolonema in the mature interphase nucleolus. $\times 26,400$



DISCUSSION

Sirlin: How do you account for the discrepancy in your present results and those you already reported in *Vicia faba* concerning the mode of formation of the nucleolus at telophase? May it not be that somewhere you are missing rapid transformation or aggregation of the interchromosomal material into the first observable nucleolus that you see at the organizer?

Chouinard: In the previous work with Lafontaine on *Vicia faba* (J Cell Biol 17: 167-201, 1963), the very earliest stages of nucleolus development at telophase were either not observed or not recognized; the youngest telophase nucleoli seen, moreover, already showed under electron microscopy the main structural features of mature nucleoli. In the present study, the early stages of nucleolus development have been recognized and the material aggregating at the nucleolar organizer is indeed shown to exhibit ultrastructural characteristics distinct from those of the material interspersed among the telophase chromosomes. Our observations are in agreement with those recently reported by Karasaki (J Cell Biol 26: 937-958, 1965) concerning the problem of nucleolar formation in *Triturus* embryos. He has presented evidence indicating that the forming nucleoli originate as small dense, exclusively fibrous bodies within the chromatin material. These dense fibrous nucleoli enlarge during successive developmental stages by the addition of granular components 150 Å in diameter which form a layer around the fibrous core.

Kasten: I am wondering a little about the staining technique used to demonstrate the various nucleolar components at the light microscope level. In the Feulgen-methylene blue technique, the Feulgen procedure would remove RNA from the structures. This means that what you would be staining would be DNA and proteins. The carboxylic acid groups of the proteins would be expected to combine with the amino groups of methylene blue. I do not understand how you can expect to see the full morphological picture at the light microscope level minus the RNA.

Chouinard: Extraction of RNA from the structures studied is very unlikely since our material was embedded in Epon. Furthermore, methylene blue alone without the Feulgen procedure also reveals the two tinctorially distinct components present within the dense portion of the nucleolar mass.

Swift: One brief comment on this last point. The same two regions, of course, may be demonstrated in a lot of other nucleoli also, such as in *Drosophila* salivary glands, and you don't need a Feulgen reaction first. They may be distinguished on 1 or 2 micron Epon or methacrylate sections just with methylene blue or azure B, by means of their differences in RNA concentration.

I was pleased you concluded that the nucleolus does not form from interchromosomal material. But as we concluded some time ago (Symp Molec Biol, 1958) there seem to be not two but three distinguishable RNA-containing components in telophase nuclei of onion roots. These are 1) the forming nucleoli, 2) the spindle material trapped between chromosomes, and 3) a series of dense inclusions which may be detected at numerous sites along the late anaphase or telophase chromosomes. Although fraction 2) disintegrates and, as you have indicated, apparently is not involved in nucleolar formation, we know very little about fraction 3). We suggested it represents sites where RNA synthesis is initiated along the chromosome, and as such may have something to do with the nucleolus in terms of chromosomal-nucleolar interaction.

Chouinard: In *Allium cepa*, the technical procedures used so far have not allowed us to reveal more than two ultrastructurally distinct fractions within the forming telophase nucleus. The first of these fractions is interchromosomal and often con-

tains imprisoned spindle remnants or even cytoplasmic organelles. The second fraction is found exclusively within the growing nucleolus at the nucleolar organizer sites.

Tandler: Evidence for the existence of a third silver-reducing fraction within the telophase nucleus has been presented by me (Exp Cell Res 17: 560-564, 1959) and also by Swanson-Beck (Exp Cell Res 28: 406-418, 1962) who showed that a nucleolar fluorescent antibody appears along all the chromosomes at telophase.

Nucleolar-Chromosomal Interaction in Microspores of Maize^{1, 2}

HEWSON SWIFT and BARBARA JEAN STEVENS,^{3, 4}
Whitman Laboratory, University of Chicago, Chicago, Illinois

SUMMARY

Nucleoli and nucleolus-like bodies were studied in microspores from two translocation stocks of maize (T6-9a and T6-9b) by basic dye binding and electron microscopy. Nucleoli from normal microspores possess a uniformly filamentous fine structure in early interphase, but by midinterphase develop peripheral particles arranged in a thread-like (nucleolonemal) structure. The divided organizer region of T6-9a produces two small normal-looking nucleoli. Nucleoli of duplication-deficient nuclei (6, 6° nuclei of T6-9b) possess a nucleolonemal structure, but contain abnormal peripheral particles larger and more irregular than in normal cells. Nuclei that lack organizers entirely possess numerous basophilic spherical bodies with a

finely filamentous structure, closely resembling the interior component of normal nucleoli. These bodies are distinguishable from true midinterphase nucleoli by the absence of peripheral particles. Both normal and abnormal microspore nuclei contain a number of other basophilic inclusions, some granular and some lamellar in structure. The accumulation of nucleolus-like bodies in anucleolate cells is interpreted as an indication that the organizer locus normally interacts with other sites of RNA synthesis on the chromosomes, either in the incorporation of materials into the nucleolus, or in providing material active in the transport of chromosomal RNA from nucleus to cytoplasm.—*Nat Cancer Inst Monogr* 23: 145-166, 1967.

IN MANY cell types several RNA-containing sites can be distinguished within the nucleus. With standard cytochemical methods, such as autoradiography and basic dye binding, RNA can be detected in the nucleolus and in various chromatin-containing regions. In favorable tissues, such

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5-10, 1965.

² Supported by grants from the National Science Foundation and the U.S. Public Health Service.

³ Dr. Stevens was supported by U.S. Public Health Service Training Grant GM-150 from the National Institute of General Medical Sciences.

⁴ We wish to express our appreciation to Mr. J. Galinis for his help in growing the plants. The valuable technical assistance of Mrs. Betty Jane Adams and Mr. Chris Chou is also gratefully acknowledged.

as dipteran salivary glands, RNA can also be demonstrated in the nucleoplasm and in multiple sites along the chromosomes. In certain classical studies on the nucleolus, it was concluded that the chromosomal and nucleolar components were somehow interrelated. Observations on mitotic cells suggested, for example, that the origin of the nucleoli in telophase involves the collection of nonspecific fragments of matrix material sloughed from all the chromosomes (1-3). This concept was strongly supported by the work of McClintock (4) on maize microspores bearing translocations of the nucleolar chromosome. As every student of cytogenetics knows, these elegant studies showed that a translocation heterozygote, involving the nucleolar chromosome 6 and chromosome 9, was capable of segregating to produce microspores deficient in the nucleolar organizer region. When the organizer was largely or entirely lacking, the early interphase nuclei contained, instead of the usual single large nucleolus, a number of smaller nucleolus-like bodies. It was logical to conclude that, without the nucleolus-forming site on chromosome 6 to "organize" the nucleolar substance, the matrix material remained disorganized and dispersed.

We became interested in this problem some 10 years ago, in the course of studies on the nuclei of heavily irradiated onion roots (5). This material contained numerous micronuclei, produced by acentric fragments which lagged on the mitotic spindle, and formed their own small nuclei in interphase. Many of these micronuclei contained small nucleolus-like bodies, but many failed to form RNA-containing inclusions of any kind. It was thus apparent that nucleolar substance was not the product of every piece of telophase chromosome, as might be expected by the matrix hypothesis. A similar conclusion was reached by Crosby (6) in the case of micronuclei produced by unpaired extra chromosomes in aneuploid strains of wheat. If the micronucleus-forming extra chromosome carried a nucleolus organizer, it formed a typical nucleolus, but no nucleoli were produced by chromosomes that lacked the organizer loci. We also studied normal telophase nuclei, utilizing azure B-deoxyribonuclease and fast green-chromic acid staining. These observations convinced us that much of the "matrix" material, which we considered to contain spindle remnant and adjacent areas of cytoplasm, although it became partly entrapped within the forming telophase nucleus, eventually disintegrated and was not directly incorporated into the new nucleolus. A similar conclusion has been expressed by Chouinard in this Symposium (7). These observations argued against the hypothesis that chromosomal matrix contributed substantially to the nucleolus, and that the role of the nucleolus-forming site on the chromosome was merely one of "organization."

The matrix hypothesis of nucleolar origin is also refuted by a large number of studies that indicate the nucleolus possesses an active role as an interphase site of RNA synthesis. The interphase variations in nucleolar size with variation in cell growth rate were early pointed out

by Heitz (8) in regenerating mosses and Hyden (9) in axon regeneration. In the studies of Lin (10) on maize, and Longwell and Svihla (11) on wheat, the volume of nucleolar material in the nucleus was shown to be substantially increased when extra organizers were added to the genome. Numerous autoradiographic studies have also demonstrated that the nucleolus actively incorporates labeled RNA precursors (12-14).

It has also been stressed that the RNA-containing components of chromatin may show synthetic characteristics different from those of the nucleolus. For example, in grasshopper spermatogenesis, nucleolar RNA decreases in amount in early prophase, when chromosomal RNA levels remain high (15). Das, in this Symposium (16), has described the active incorporation of H^3 -cytidine into chromosomal RNA in grasshopper spermatocytes; thus the chromosomal synthesis clearly proceeds independently of that in the nucleolus. On the other hand, in certain cell types, such as mid third instar *Drosophila* salivary glands, the nucleolus shows much more rapid incorporation than the chromosomes (17, 18), and thus the nucleolus organizer also appears capable of independent synthesis of RNA. Such observations point to the obvious conclusion that the genome of a cell contains numerous sites for RNA synthesis, including the organizer locus, and that most, if not all, of the nucleolar substance is synthesized at the organizer rather than forming as an aggregation of material made elsewhere on the chromosomes. The point that the nucleolus organizer locus is only one of a number of independent RNA-synthesizing loci along the chromosome has been made in this Symposium by Pelling (19).

The relative independence of nucleolus and chromosomal RNA, and the ubiquitous presence of nucleoli in all growing or synthesizing cells seem adequately explained by the concept of the nucleolus as the site for the synthesis of ribosomal RNA. This is strongly indicated by the absence of incorporation into 18s and 28s ribosomal RNA in anucleolate *Xenopus* (20), and the annealing of *Drosophila* ribosomal RNA to DNA in proportion to the number of organizers in the genotype (21). The fact that base ratios of nucleolar RNA resemble those of the predominantly ribosomal cytoplasm (22) is an additional indication of the involvement of the nucleolus in ribosomal RNA synthesis. Probably, as stressed by Beermann (23), RNA synthesis on other sites along the chromosome represents the formation of messenger, and possibly also of transfer RNA.

This concept of the independence of organizer and other chromosomal sites in RNA synthesis, however, fails to explain the findings of McClintock (4) that diffuse nucleolus-like material forms at multiple sites if the organizer is disrupted or lacking. It seems to us that this phenomenon provides a clear indication that nucleolar organizer and chromosomal sites of RNA synthesis do not act in a completely independent fashion, despite the fact that they are apparently involved in the synthesis of different RNAs. Thus, although we can probably discard the "matrix" hypothesis of nucleolar formation, we still must consider that the organizer locus has

an important influence on the accumulation of material at other sites within the nucleus. The present study was undertaken as a step toward our understanding of the nature of this nucleolar-chromosomal interaction.

MATERIALS AND METHODS

Two field-grown stocks of maize were studied, which were obtained from the Corn Coop, University of Illinois, Urbana, Illinois, through the courtesy of Dr. E. B. Patterson. Both stocks were translocation heterozygotes, involving chromosomes 6 and 9. Stock T6-9a contained translocation 6S(79%)—9L(40%) (4), and stock T6-9b translocation 6L(10%)—9S(37%) (24). Normal chromosomes were from inbred line M14. Immature anthers were fixed in glutaraldehyde-osmium (25) at pH 6.8 and embedded in Epon. Thin sections were stained in uranyl acetate and lead hydroxide.

OBSERVATIONS

Translocation heterozygotes show a complex of four chromosomes at pachytene, which usually opens to a ring or chain at metaphase I. The complex can segregate with respect to the organizer in three different ways. In translocation T6-9b, for example, the pachytene complex contains chromosomes 6, 6⁹, 9, and 9⁶, of which 6 and 6⁹ contain the organizer loci. Segregation at anaphase I can be of three types: *alternate* (6 and 9 to one pole, 6⁹ and 9⁶ to the other); *adjacent 1*, in which homologous centromeres pass to opposite poles (6 and 9⁶ to one pole, 6⁹ and 9 to the other); or *adjacent 2*, in which homologous centromeres pass to the same pole (6 and 6⁹ to one pole, 9 and 9⁶ to the other) (26). If no crossovers occur in the interstitial segments between centromere and translocation, then alternate segregation results in four viable microspores, each with a single organizer. Adjacent 1 segregation also produces 4 microspores, each containing a nucleolar organizer, but the cells all possess major deletions and are inviable. Adjacent 2 segregation produces two microspores with 2 nucleolar organizers each, and 2 microspores lacking the organizer. All 4 cells are also inviable. Where crossovers occur in the interstitial segments, the quartet of cells may contain 2 microspores with single organizers, one with 2 and one with none; in certain cases the microspores with single organizers would be complete and viable, but in others would be deficient and abort (24). Abnormal microspores may also occur where chromosomal segregation at metaphase I is 3:1 instead of 2:2.

If acetocarmine squashes are made of whole anthers in the quartet stage, microspores may be scored for single nucleoli, two nucleoli, or none, in which case numerous "diffuse" stainable bodies occur in the nucleus. The distribution of microspore types was determined for T6-9b by Burnham (24). Of 12,388 microspores examined, 13.4% contained "diffuse" nuclei.

Almost all of these diffuse nuclei were in an adjacent 2 configuration, with a total of 26.8% of the quartets showing two diffuse and two binucleolate nuclei. Only 0.5% showed a single diffuse nucleus per quartet, resulting from interstitial crossovers. The number of adjacent 2 quartets is close to the 25% expected on the basis of equal likelihood of adjacent 1 and adjacent 2 segregation. Also, pollen abortion was 50.2%, matching a 1:1 ratio of alternate (viable) and adjacent (nonviable) segregation.

In translocation T6-9a the situation is more complex, since the break in chromosome 6 passes through the organizer. The distribution of microspore types was determined by McClintock (4). Viable microspores, arising from alternate segregation, contained single nucleoli where the chromosomal complement was normal (6, 9) and either two nucleoli or a single fusion nucleolus in nuclei carrying the translocation chromosomes (6⁹, 9⁶). Interestingly enough, microspores containing chromosomes 6 and 9⁶ (type 4) possessed diffuse nuclei even though a normal nucleolus organizer was present on an intact chromosome 6, in addition to an organizer fragment on chromosome 9⁶. Nuclei of this type obviously possess a duplication for the satellite on chromosome 6, and a deletion of two thirds of the long arm of chromosome 9 and are thus inviable.

Light micrographs of 1 μ Epon sections are shown in figures 1 and 2. Figure 1 shows a normal microspore part way through interphase and the normal cytoplasmic vacuolation process. The exine is well developed. The nucleolus stains typically purple with azure B which is characteristic of the presence of RNA (27). By midinterphase the nucleoli show a rim of more intensely staining material, often enlarged on one side to form a cap. Figure 2 shows early microspores in the quartet stage; in the upper cell the section passes through one of two nucleoli of a 6, 6⁹ nucleus of T6-9b, possessing a prominent cap at the right margin. The lower cell possesses a "diffuse" 9, 9⁶ nucleus, showing several small basophilic inclusions, staining purple with azure B. It should be noted that small basophilic inclusions are characteristic of all microspore nuclei at this stage, though they appear more prominent in the diffuse nuclei.

A normal microspore at midinterphase is shown in figure 3, which is from the same anther as the cell in figure 1. The nucleolus is shown enlarged in figure 4, and the basophilic cap region is further magnified in figure 7. Most of the nucleolar mass consists of the finely filamentous or nonparticulate region. A narrow ring of particles surrounds the nucleolus and the particulate component is prominent in the cap. This region also possesses the threadlike nucleolonemal structure characteristic of many nucleoli. Several small nucleolar vacuoles are present. A small nucleolus-like body, closely resembling the inner nucleolar mass in its structure, is shown to the left of the nucleolus in figures 3 and 4.

The particulate component of nucleoli from normal microspores was not seen in normal quartet and early interphase nuclei. Apparently the particles arise only after the cells have progressed to the midinterphase stage. The margin of a normal nucleolus in the early microspore stage

is shown in figure 6. The nucleolus contains only the finely filamentous component and particles are lacking.

An early microspore from T6-9b is shown in figure 8. The nucleus contains four large inclusions and several smaller adjacent structures. Part of a diffuse nucleus from T6-9a is shown at higher magnification in figure 9. This cell probably contains chromosomes 6 and 9⁶. The nucleolus-like bodies possess a variety of different morphologies, but particulate margins characteristic of nucleoli in normal microspores were not seen. The structure of most of these inclusions is finely filamentous, closely resembling the inner component of normal nucleoli. Irregular filamentous or particulate material often occurs at the edges of these bodies, but the regular particulate component of normal nucleoli is lacking (figs. 5, 8, and 9). In some instances they contain vacuoles and possess a peripheral nucleolonemal structure.

Nucleoli are also abnormal in the binucleolate microspores of T6-9b. These nucleoli possess basophilic caps and threadlike structures and also contain vacuoles (figs. 2, 10, and 11). In some instances they had an outer particulate component that was larger (30-40 m μ in diameter), less dense, and less regular than the peripheral particles of normal nucleoli. Most of the nucleolar mass, however, had the usual undifferentiated finely filamentous structure.

In 6⁹, 9⁶ nuclei of T6-9a the chromosome complement is complete and the cell is viable. The organizer locus has been divided into two portions, however, also resulting in binucleolate nuclei. In these cells both nucleoli contain typical small peripheral particles and are indistinguishable from normal nucleoli except in their smaller size. It is of interest that each portion of the divided organizer is capable of forming a normal-looking nucleolus.

In addition to the dense, more or less spherical nucleolus-like bodies, a number of other basophilic inclusions were seen in both normal and abnormal microspores. These included granular masses interspersed with a more uniform material of lower electron density, as shown in figure 9, irregular granular and filamentous masses, as in figure 14, and compound lamellate bodies containing layers of dense material with a structure suggesting periodicity, which were often associated with homogeneous areas of irregular outline (figs. 12 and 13). At present the nature of these inclusions is unknown, although they all apparently contain RNA. It is postulated that they represent ribonucleoprotein aggregates formed at chromosomal loci other than the organizer. They may thus be similar to the heterogeneous RNA-containing inclusions found in the primary spermatocytes of *Drosophila* (28). Although these irregular inclusions have been found in both normal and abnormal microspores, as yet we have not been able to ascertain whether they are larger or more prevalent in diffuse nuclei.

It should be emphasized that approximately half of the microspores in heterozygous translocation stocks such as T6-9b may be expected to abort,

including all cells with binucleolate and diffuse nuclei. Thus it is difficult to determine which characteristics of the nucleolus-like bodies reported here are a direct consequence of absent or disrupted organizers, and which may be the indirect consequence of other abnormalities associated with the deficient genotype and its influence on cell metabolism.

DISCUSSION

In the absence of the nucleolar organizer region on chromosome 6, a large number of ribonucleoprotein inclusions accumulate within the microspore nucleus. As pointed out by McClintock (4), there is an obvious reciprocal relationship between the presence of a typical nucleolus and the presence of prominent nucleolus-like inclusions. We have hoped to shed some light on the nature of this nucleolar-chromosomal interaction. So far we have concluded the following: Nucleoli in normal early microspores of maize are homogeneous and finely filamentous in structure, without a particulate peripheral component. Later, in midinterphase, peripheral particles are visible, usually associated with a threadlike nucleolonemal structure on one side of the nucleolus. In diffuse nuclei, which lack a functional nucleolus organizer, numerous dense, RNA-containing spherical inclusions are apparent. Most of these closely resemble the nucleolus of the early microspores in appearance but apparently never develop a normal peripheral zone of particles. In both normal and abnormal microspores, other RNA-containing inclusions are also present, some of which possess a coarsely granular and others a lamellar structure. The finely filamentous central component of the nucleolus apparently also forms first in cleaving eggs of *Triturus* (29), with the particulate margin arising later. In nuclei of anucleolate *Xenopus* larvae, nucleolus-like bodies are also present, with essentially a uniform filamentous structure (30), though the presence of a particulate component has also been described (31).

The morphological picture of normal and abnormal microspores suggests that the 'extra' nucleolus-like bodies in diffuse nuclei are not true nucleoli, being distinguishable at least by midinterphase by the absence of particles, in contrast to true functional nucleoli which develop a particulate margin. The situation is complicated, however, by the fact that nucleoli in the deficient and nonviable 6, 6^o microspores of T6-9b, even though they develop from normal nucleolar organizers, do not possess a normal particulate periphery. The large, irregular particulate component of these bodies is not found in either the normal nucleoli or the diffuse nucleolus-like bodies. Normal nucleoli appear only in 6, 9 or 6^o, 9^o microspores, which bear complete genomes and are fully viable. Since all diffuse nuclei in our material also possess chromosomal duplications and deletions, certain characteristics of the nucleolus-like bodies may be due to the absence of the organizer, but others may be associated with additional effects of the abnormal genome.

There is an obvious question as to why diffuse material should accumulate in anucleolate nuclei. Explanations of two kinds may be suggested: 1) The cell contains a number of secondary organizer loci that are normally repressed in the presence of a functional nucleolus. They are, however, capable of derepression, and of substituting for the nucleolus in the absence of the organizer (4, 5). 2) The nucleolus-like bodies are not "secondary nucleoli," but rather the synthetic products of specific chromosomal loci normally interacting with the nucleolus. This interaction may involve either (a) the *contribution* of materials to the nucleolus, or (b) nucleolar products may be involved in the *removal* of chromosomal products, in their transferral to the cytoplasm. In either case, chromosomal ribonucleoprotein material would accumulate in the absence of the organizer.

The first alternative, that the diffuse bodies represent true secondary nucleoli, can be eliminated. The nucleolus-like bodies in our material are morphologically distinguishable from true nucleoli. In anucleolate *Xenopus* no 18s or 28s RNA is made (20), and thus no secondary loci can assume the function of ribosomal RNA synthesis. In *Drosophila*, ribosomal RNA anneals specifically to organizer loci (21), and thus there is no evidence for the presence of secondary binding sites which would represent auxiliary organizer loci.

The second alternative postulates that the basophilic bodies in diffuse nuclei represent the synthetic products of other non-nucleolar loci. It is now well established that RNA may form at multiple sites along the chromosomes, as in the puffs of dipteran salivary glands (23, 32), and the loops of lampbrush chromosomes in vertebrate oocytes (33) or *Drosophila* spermatocytes (28). Also, what appear to be chromosomal RNA-containing inclusions appear along the chromosomes of onion and grasshopper cells at anaphase and telophase (5, 34).

It is of interest to examine the cytological evidence for hypothesis 2a) (above) that the products of several chromosomal loci, in addition to the organizer, contribute substantially to the nucleolar mass. In the studies of Lin (10), extra nucleolus organizers added to the maize genome on small B chromosomes caused a significant increase in nucleolar volume and nucleoprotein content at pachytene. Nucleolar mass, however, did not precisely double with a doubling of organizer number but showed a smaller increment. Lin concluded that a major portion, but not all, of the nucleolar mass was the product of the organizer loci. He suggested that a certain component may be the product of other chromosomal regions.

There are several other aspects of nucleolar metabolism that are as yet poorly understood but also point to a possible complexity of nucleolar origin. Little is known, for example, concerning the source of nucleolar proteins. Autoradiographic data with labeled amino acids (35, 36) fail to show the active incorporation that might be expected if the nucleolus were the site of ribosomal protein synthesis. Indeed the amino acid in-

corporation is lower in early interphase nuclei of *Vicia* roots, when nucleoli are forming, than during other times in the mitotic cycle (36). Although little can be concluded about the site of synthesis of nucleolar proteins, there is a strong possibility that they are not formed *in situ* but are contributed from other locations in the cell.

The complexity of nucleolar RNA incorporation patterns, well illustrated by Kleinfeld in this Symposium (37), also argues against the simple concept of the nucleolus solely as the product of the organizer locus. The presence of at least two different RNA fractions, with markedly different turnover times, would seem to be indicated by these data. Evidence for the existence of slow and fast nucleolar RNA fractions from starfish oocytes has been presented by Vincent (38), and Amano *et al.* (39) believed that their RNA incorporation data for mouse tissues would best be fitted by postulating three distinct components. Although ribosomal RNA is itself heterogeneous, the fact that both 18s and 28s components apparently originate from 45s molecules (40) makes it unlikely that the heterogeneity of ribosomal RNA is directly related to the heterogeneity of nucleolar incorporation. It seems likely, then, that the nucleolus contains ribosomal RNAs, but at least one additional fraction of unknown origin and function. The possible presence of soluble RNA in the nucleolus is controversial and is discussed elsewhere in this Symposium.

Hypothesis 2b) could also explain the formation of nucleolus-like bodies in anucleolate nuclei. This states that the products of nucleolar synthesis may be needed to transport RNA-containing components from sites on the chromosomes out to the cytoplasm. In the absence of a true nucleolus, these chromosomal RNA fractions thus would accumulate in abnormal amounts at the sites of their formation. Several different findings support this concept. When chromosomal RNA forms at a specific site on a polytene chromosome of *Drosophila* or *Chironomus*, the puffed region shows a rapid precursor incorporation (41, 42). This is a strong suggestion that the puff represents a site of synthesis of chromosomal RNA. Puff formation involves not only the accumulation of RNA, but also of nonhistone protein (32). Much of the substance of the puff is in the form of ribonucleoprotein granules (43). These granules are usually larger and less regular than ribosomes, and are capable of leaving the chromosome and entering the nuclear sap and apparently of passing through the annuli of the nuclear envelope into the cytoplasm (44). Autoradiographic studies with labeled amino acids indicate that the nonhistone puff protein does not become strongly labeled during puff formation (45, 46). It thus is pre-formed and merely accumulates at the puffed locus. Its original site of synthesis is unknown.

It has been suggested by McConkey and Hopkins (47), on the basis of fractionation studies on HeLa cells, that the first step in the transport of messenger RNA from chromosome to cytoplasm may involve a complex between messenger and the smaller (45S) subribosomal particle. Com-

plexes between ribosomal subunits and messenger RNA have also been found in the cytoplasm by other investigators (48, 49), and their nature has been discussed by Perry (50) in this Symposium. The presence of a subribosomal transport system for chromosomal RNA would explain the accumulation of nonhistone proteins at the puff sites in polytene chromosomes. It would also provide a convenient explanation for the accumulation of chromosomal RNA in anucleolate nuclei.

Our studies on the fine structure and cytochemistry of maize microspores are still in progress. We can come to the tentative conclusion, however, that the changes in chromosomal RNA fractions produced in anucleolate nuclei suggest that a close interaction exists between the nucleolus organizer and the sites of chromosomal RNA synthesis. This interaction may involve either the contribution of ribonucleoprotein material from sites of formation on the chromosomes to the nucleolus, or conversely the contribution of material such as ribosomal subunits from the nucleolus to the chromosomes, in the possible transport to the cytoplasm of messenger RNA. An extensive mixing and randomization of nuclear RNA fractions, however, is obviously refuted by the demonstrated differences in base ratios (22) and incorporation patterns (17) of nucleolus and chromosomal regions. Although the nature of this nucleolar-chromosomal interaction is far from clear, it can serve to indicate that certain problems concerning nucleolar function are complex and doubtless involve the interdependence of different RNA-forming sites within the nucleus.

RESUMEN

Se han estudiado cuerpos semejantes a nucleolos o cuerpos nucleolares en microsporos de dos cepas translocacias de maíz (T6-9a y T6-9b) mediante tinción con colorante básico y microscopía electrónica. Los nucleolos de microsporos en interfase temprana poseen una fina estructura uniformemente filamentososa, pero en la interfase (nucleolonemal). La región dividida del organizador de T6-9a produce dos pequeños nucleolos de apariencia normal. Los nucleolos de núcleos de duplicación deficiente (núcleos 6, 6^o de T6-9b) poseen una estructura nucleolonemal, pero contienen partículas periféricas anormales, mayores y más irregulares que en las células normales. Los núcleos que carecen por completo de organizadores, poseen numerosos cuerpos esféricos basofílicos con una estructura finamente filamentososa muy parecida a los componentes interiores de los nucleolos normales. Estos cuerpos se distinguen de los verdaderos nucleolos de interfase media por la ausencia de partículas periféricas. Tanto los núcleos de microsporos normales como los anormales contienen cierto número de otras inclusiones basofílicas, algunas granulares y otras compuestas de láminas, en estructura. La acumulación de cuerpos semejantes a cuerpos nucleolares en las células anucleoladas se interpreta como una indicación de que el locus organizador normalmente tiene interacción con otros sitios de síntesis del ARN en los cromosomas, ya sea en la incorporación de materiales dentro del nucleolo o en la provisión de material activo en el transporte de ARN cromosómico del núcleo al citoplasma.

REFERENCES

- (1) HEITZ, E.: Die Ursache der gesetzmässigen Zahl, Lage, Form, und Grösse pflanzlicher Nukleolen. *Planta* 12: 775-844, 1931.
- (2) RATTENBURY, J. A., and SERRA, J. A.: Types of nucleolus reconstitution in telophase and the question of the "nucleolus organizer." *Port Acta Biol, Series A*, 3: 239-260, 1952.
- (3) LAFONTAINE, J. G., and CHOUINARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167-201, 1963.
- (4) MCCLINTOCK, B.: The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
- (5) SWIFT, H.: Studies on nucleolar function. *In A Symposium on Molecular Biology* (Zirkle, R. E., ed.). Chicago, Univ Chicago Press, 1959, pp 266-303.
- (6) CROSBY, A. R.: Nucleolar activity of lagging chromosomes in wheat. *Amer J Bot* 44: 813-822, 1957.
- (7) CHOUINARD, L. A.: Nucleolar architecture in root meristematic cells of *Allium cepa*. *Nat Cancer Inst Monogr* 23: 125-143, 1966.
- (8) HEITZ, E.: Das Verhalten von Kern und Chloroplasten bei der Regeneration. *Z Zellforsch* 2: 69-86, 1925.
- (9) HYDEN, H.: Protein metabolism in the nerve cell during growth and function. *Acta Physiol Scand* 6, Suppl 17: 1-136, 1943.
- (10) LIN, M.: Chromosomal control of nucleolar composition in maize. *Chromosoma* 7: 340-370, 1955.
- (11) LONGWELL, A., and SVIHLA, G.: Specific chromosomal control of the nucleolus and of the cytoplasm in wheat. *Exp Cell Res* 20: 294-312, 1960.
- (12) FICQ, A.: Étude autoradiographique du métabolisme des protéines et des acides nucléiques au cours de l'oogénèse chez les batraciens. *Exp Cell Res* 9: 286-293, 1955.
- (13) GOLDSTEIN, L., and MICOV, J.: Nuclear-cytoplasmic relationships in human cells in tissue culture. III. Autoradiographic study of interrelation of nuclear and cytoplasmic ribonucleic acid. *J Biophys Biochem Cytol* 6: 1-6, 1959.
- (14) KLEINFELD, R. G., and VON HAAM, E.: Nucleic acid metabolism in regenerating rat liver using cytidine H³. *Ann Histochim* 7-3: 89-96, 1962.
- (15) SWIFT, H., REBHUN, L., RASCH, E., and WOODARD, J.: The cytology of nuclear RNA. *In Cellular Mechanisms in Differentiation and Growth* (Rudnick, D., ed.). Princeton, Princeton Univ Press, 1956, pp 45-59.
- (16) DAS, N., and ALFERT, M.: Nucleolar RNA synthesis during mitotic and meiotic prophase. *Nat Cancer Inst Monogr* 23: 337-351, 1966.
- (17) McMASTER-KAYE, R.: The metabolic characteristics of nucleolar, chromosomal, and cytoplasmic ribonucleic acid of *Drosophila* salivary glands. *J Biophys Biochem Cytol* 8: 365-378, 1960.
- (18) SWIFT, H.: The histones of polytene chromosomes. *In The Nucleohistones* (Bonner, J., and Ts'o, P., eds.). San Francisco, Holden-Day, Inc., 1964, pp 169-183.
- (19) PELLING, C., and BEERMANN, W.: Diversity and variation of the nucleolar organizing regions in Chironomids. *Nat Cancer Inst Monogr* 23: 393-409, 1966.
- (20) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (21) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.

- (22) EDSTRÖM, J. E., and BEERMANN, W.: The base composition of nucleic acids in chromosomes, puffs, nucleoli and cytoplasm of *Chironomus* salivary gland cells. *J Cell Biol* 14: 371-379, 1962.
- (23) BEERMANN, W.: Structure and function of interphase chromosomes. In *Genetics Today* (Geerts, S. J., ed.). New York and London, Pergamon Press, 1963, vol 2, pp 375-384. (Proc XI Intern Congress Genetics.)
- (24) BURNHAM, C. R.: Chromosome segregation in translocations involving chromosome 6 in maize. *Genetics* 35: 446-481, 1950.
- (25) LEDBETTER, M. C., and PORTER, K. R.: A "microtubule" in plant fine structure. *J Cell Biol* 19: 239-250, 1963.
- (26) MCCLINTOCK, B.: *Neurospora*. I. Preliminary observations of the chromosomes of *Neurospora crassa*. *Amer J Bot* 32: 671-678, 1945.
- (27) FLAX, M., and HIMES, M.: Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol Zool* 25: 297-311, 1952.
- (28) HESS, O., and MEYER, G. F.: Chromosomal differentiations of the lampbrush type formed by the Y chromosome in *Drosophila hydei* and *Drosophila neohydei*. *J Cell Biol* 16: 527-539, 1963.
- (29) KARASAKI, S.: Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J Cell Biol* 26: 937-958, 1965.
- (30) JONES, K. W.: The role of the nucleolus in the formation of ribosomes. *J Ultrastruct Res* 13: 257-262, 1965.
- (31) HAY, E. D., and GURDON, J. B.: Fine structure of the nucleolus in developing embryos of the 0 nu and 1 nu *Xenopus laevis* mutant. *Anat Rec* 154: 355, 1966.
- (32) SWIFT, H.: Nucleic acids and cell morphology in dipteran salivary glands. In *The Molecular Control of Cellular Activity* (Allen, J. M., ed.). New York, McGraw Hill, 1962, pp 73-125.
- (33) GALL, J. G., and CALLAN, H. G.: H³ uridine incorporation in lampbrush chromosomes. *Proc Nat Acad Sci USA* 48: 562-570, 1962.
- (34) STEVENS, B. J.: The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J Cell Biol* 24: 349-368, 1965.
- (35) LEBLOND, C. P., and AMANO, M.: Synthetic processes in the cell nucleus. IV. Synthetic activity in the nucleolus as compared to that in the rest of the cell. *J Histochem Cytochem* 10: 162-174, 1962.
- (36) WOODARD, J. W., RASCH, E., and SWIFT, H.: Nucleic acid and protein metabolism during the mitotic cycle in *Vicia faba*. *J Biophys Biochem Cytol* 9: 445-462, 1961.
- (37) KLEINFELD, R. G.: Altered patterns of RNA metabolism in liver cells following partial hepatectomy and thioacetamide treatment. *Nat Cancer Inst Monogr* 23: 369-378, 1966.
- (38) VINCENT, W. S.: Heterogeneity of nuclear ribonucleic acid. *Science* 126: 306-307, 1957.
- (39) AMANO, M., LEBLOND, C. P., and NADLER, N. J.: Radioautographic analysis of nuclear RNA in mouse cells revealing three pools with different turnover times. *Exp Cell Res* 38: 314-340, 1965.
- (40) SCHERRER, K., and DARNELL, J. E.: Sedimentation characteristics of rapidly labeled RNA from HeLa cells. *Biochem Biophys Res Commun* 7: 486-490, 1962.
- (41) RUDKIN, G. T., and WOODS, P.: Incorporation of H³ cytidine and H³ thymidine into giant chromosomes of *Drosophila* during puff formation. *Proc Nat Acad Sci USA* 45: 997-1003, 1959.
- (42) PELLING, C.: Chromosomal synthesis of ribonucleic acid as shown by incorporation of uridine labeled with tritium. *Nature (London)* 184: 655-656, 1959.
- (43) SWIFT, H., ADAMS, B. J., and LARSEN, K.: Electron microscope cytochemistry of nucleic acids in *Drosophila* salivary glands and *Tetrahymena*. *J Roy Mic Soc* 83: 161-167, 1964.

- (44) STEVENS, B. J., and SWIFT, H.: RNA transport from nucleus to cytoplasm in *Chironomus* salivary glands. In press. J Cell Biol, 1966.
- (45) CLEVER, U.: Puffing of giant chromosomes of Diptera and the mechanism of its control. In The Nucleohistones (Bonner, J., and Ts'o, P., eds.). San Francisco, Holden-Day, Inc., 1964, pp 317-334.
- (46) RITOSSA, F. M., PULITZER, J. F., SWIFT, H., and VON BORSTEL, R. C.: On the action of ribonuclease in salivary gland cells of *Drosophila*. Chromosoma 16: 144-151, 1965.
- (47) McCONKEY, E. H., and HOPKINS, J. W.: Subribosomal particles and the transport of messenger RNA in HeLa cells. J Molec Biol 14: 257-270, 1965.
- (48) JOKLIK, W. K., and BECKER, Y.: Studies on the genesis of polyribosomes. I. Origin and significance of the subribosomal particle. J Molec Biol 13: 496-510. II. The association of nascent messenger RNA with the 40s subribosomal particle. J Molec Biol 13: 511-520, 1965.
- (49) HENSHAW, E. C., REVEL, M., and HIATT, H. H.: A cytoplasmic particle bearing messenger ribonucleic acid in rat liver. J Molec Biol 14: 241-256, 1965.
- (50) PERRY, R. P.: On ribosome biogenesis. Nat Cancer Inst Monogr 23: 527-545, 1966.

PLATE 27

FIGURE 1.—Normal microspore in midinterphase.

FIGURE 2.—Two early microspores from a quartet of T6-9b. *Upper nucleus* shows one of two nucleoli (type 6, 6^o). *Lower nucleus* shows “diffuse” material typical of anucleolate cells (type 9, 9^o). $\times 1,500$

FIGURE 3.—Low-power electron micrograph of normal midinterphase microspore, from the same anther shown in figure 1. $\times 2,500$

FIGURE 4.—Nucleolus from cell shown in figure 3, at higher magnification. A small homogeneous inclusion is present *at left*. Such bodies are common in normal microspores. $\times 19,500$

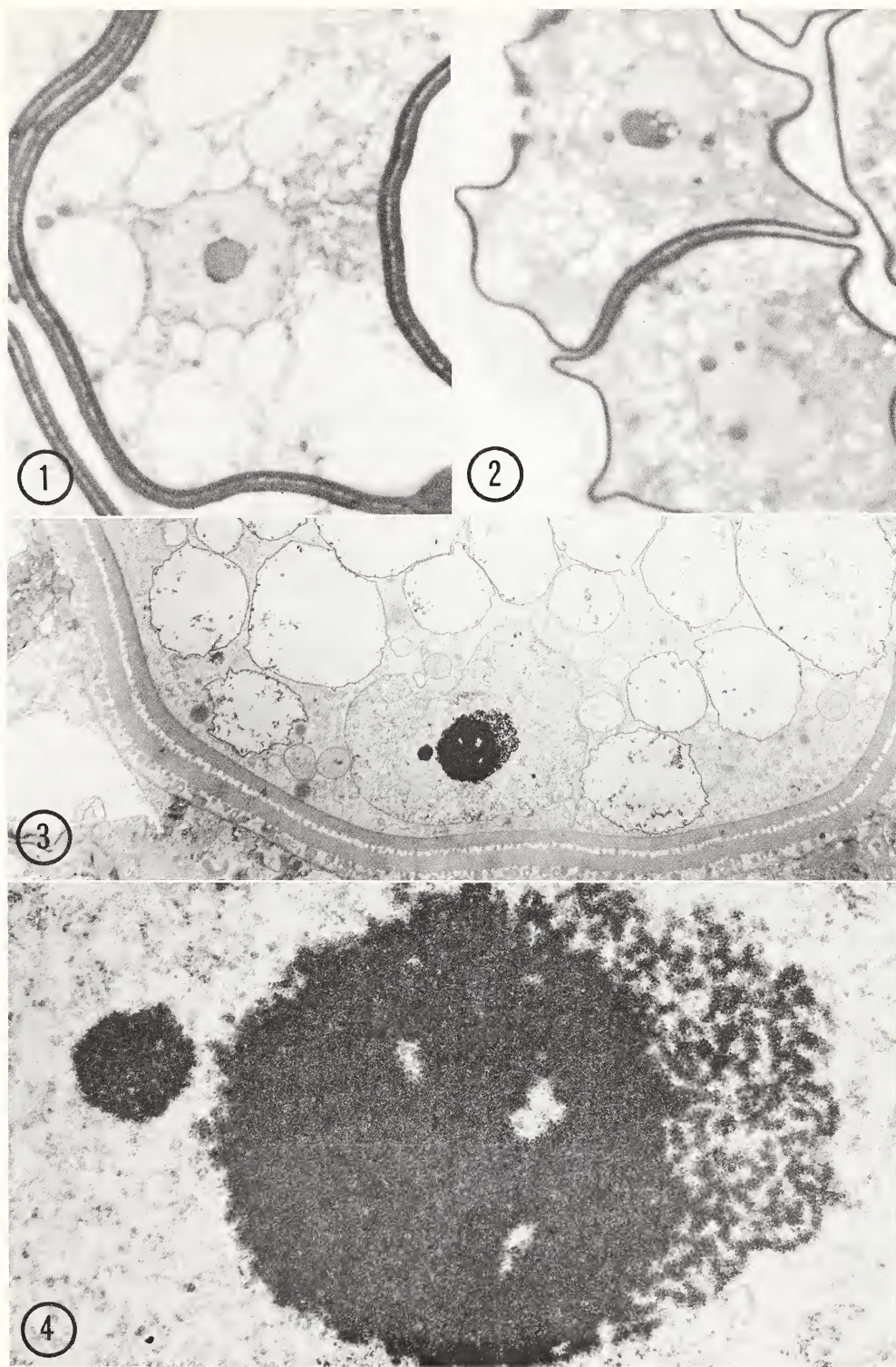


PLATE 28

FIGURE 5.—Portion of nucleolus-like inclusion from an anucleolate nucleus of T6-9b, type 9, 9^e). *Note* absence of particles in periphery. $\times 58,500$

FIGURE 6.—Nucleolus, probably normal, from an early microspore of T6-9b. Peripheral particulate component is not yet evident. $\times 58,500$

FIGURE 7.—Typical particulate and nucleolonemal structure of a normal midinterphase nucleolus enlarged from figures 3 and 4. $\times 58,500$

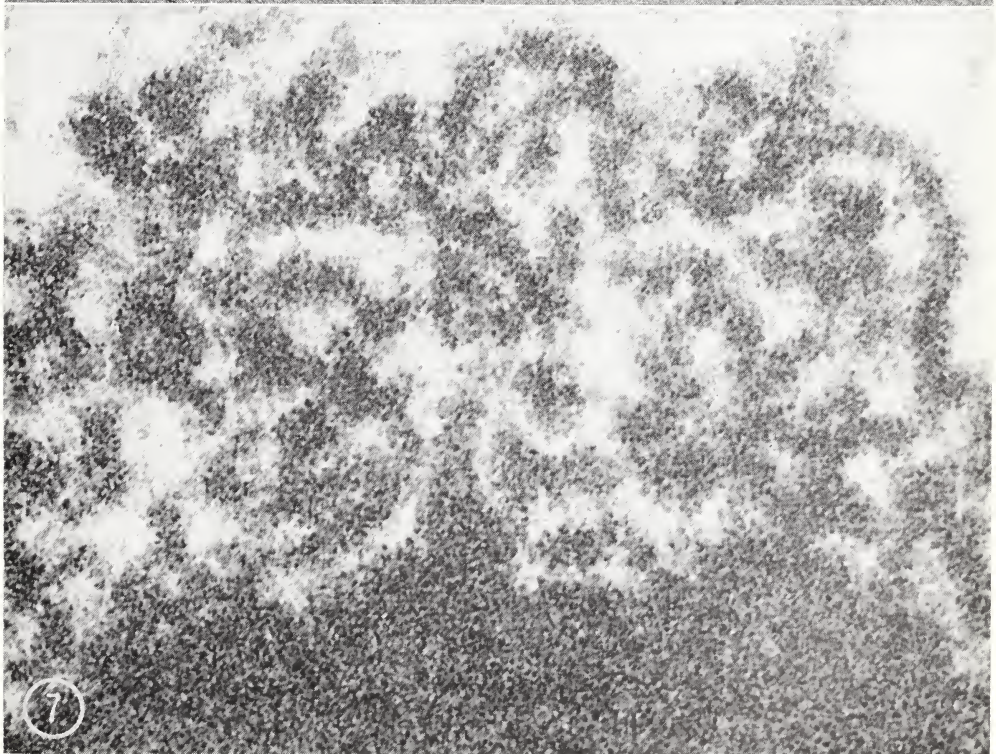
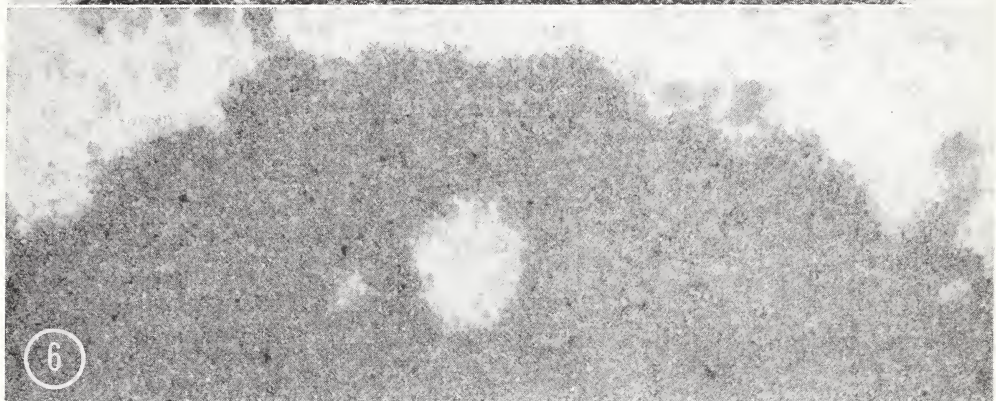
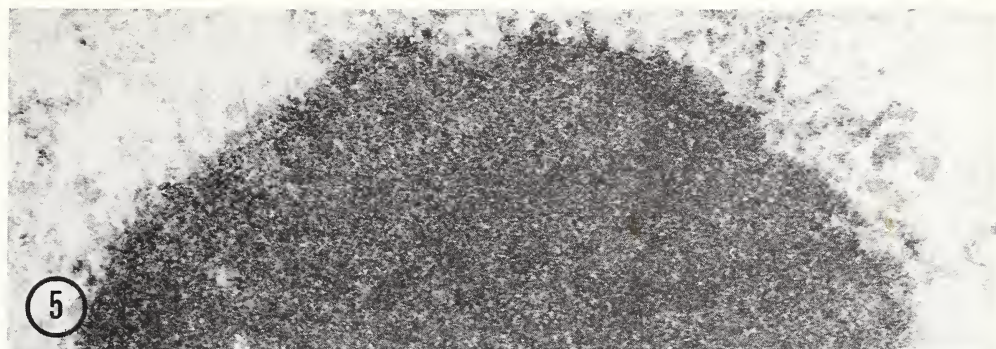


PLATE 29

FIGURE 8.—“Diffuse” nucleus (type 9, 9⁶) from same anther of T6-9b shown in figure 2, showing multiple nucleolus-like bodies. $\times 4,300$

FIGURE 9.—“Diffuse” nucleus (type 6, 9⁶) from T6-9a, showing one large nucleolus-like body *at left*, possibly the product of the normal chromosome 6 organizer which is present in the diffuse nuclei of this stock. Granular clusters (G) were found in both normal and abnormal nuclei. $\times 19,500$

FIGURE 10.—The two abnormal nucleoli of a binucleolate cell of T6-9b (type 6, 6⁹). Although this nucleus contains two organizers, it also contains a deletion for a large part of chromosome 9. The periphery and vacuoles contain large irregular particles of low electron density. $\times 43,500$

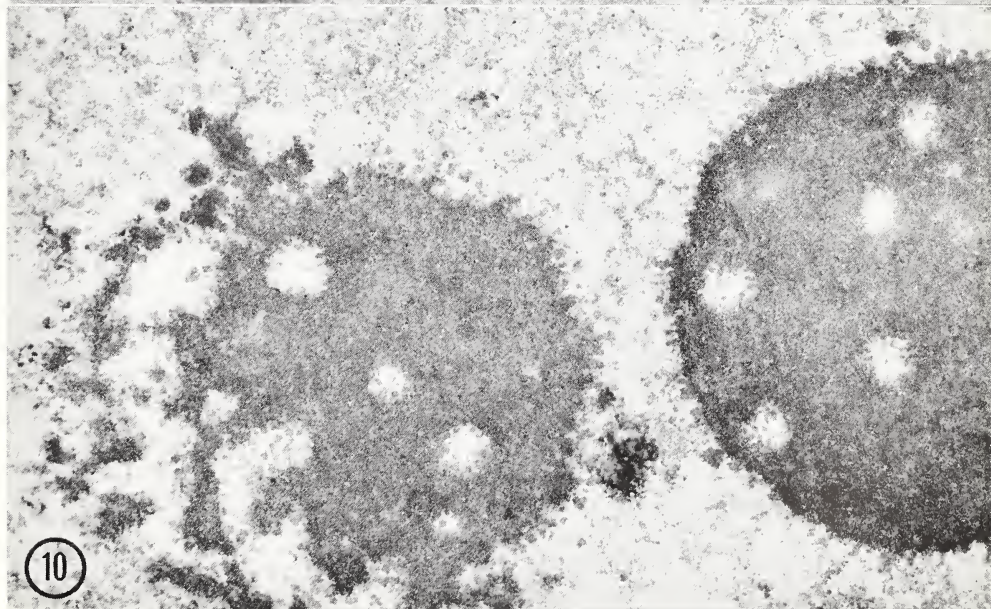
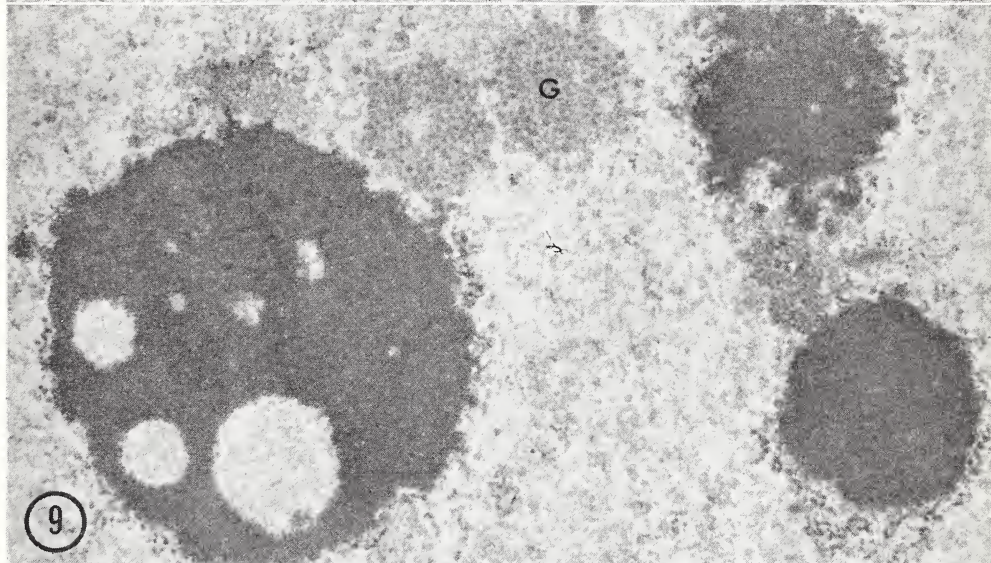
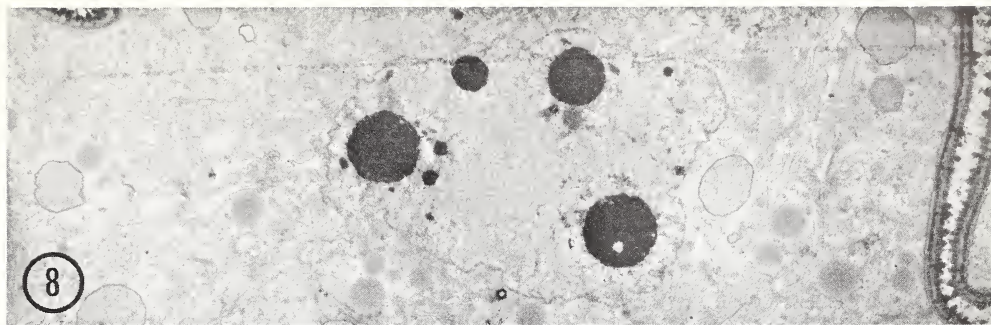


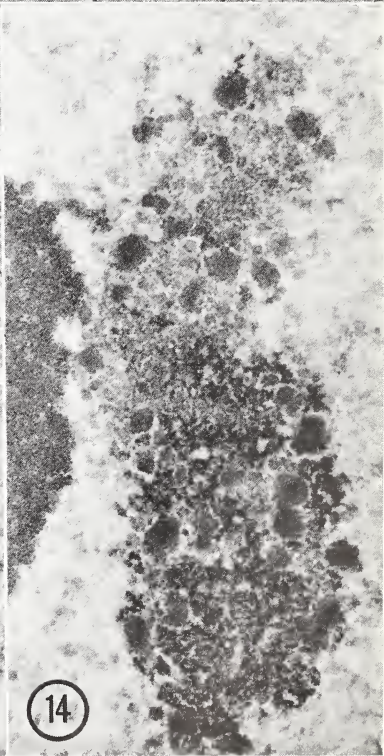
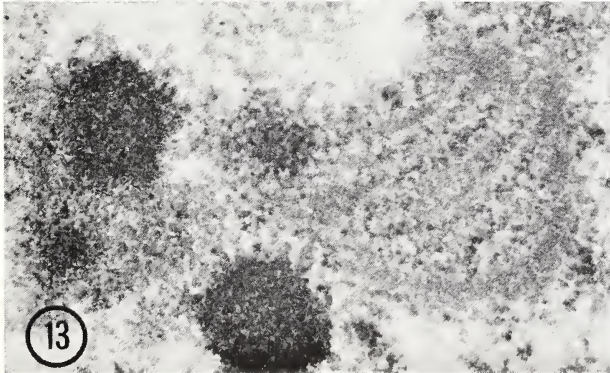
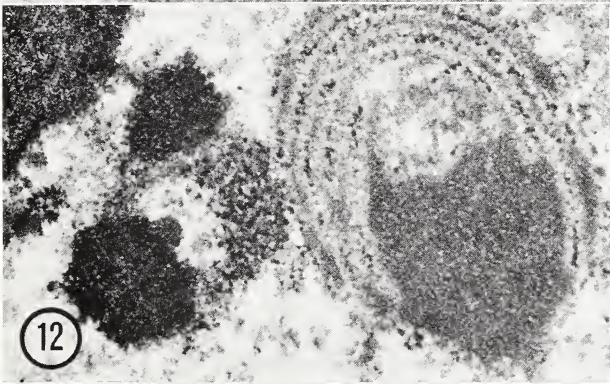
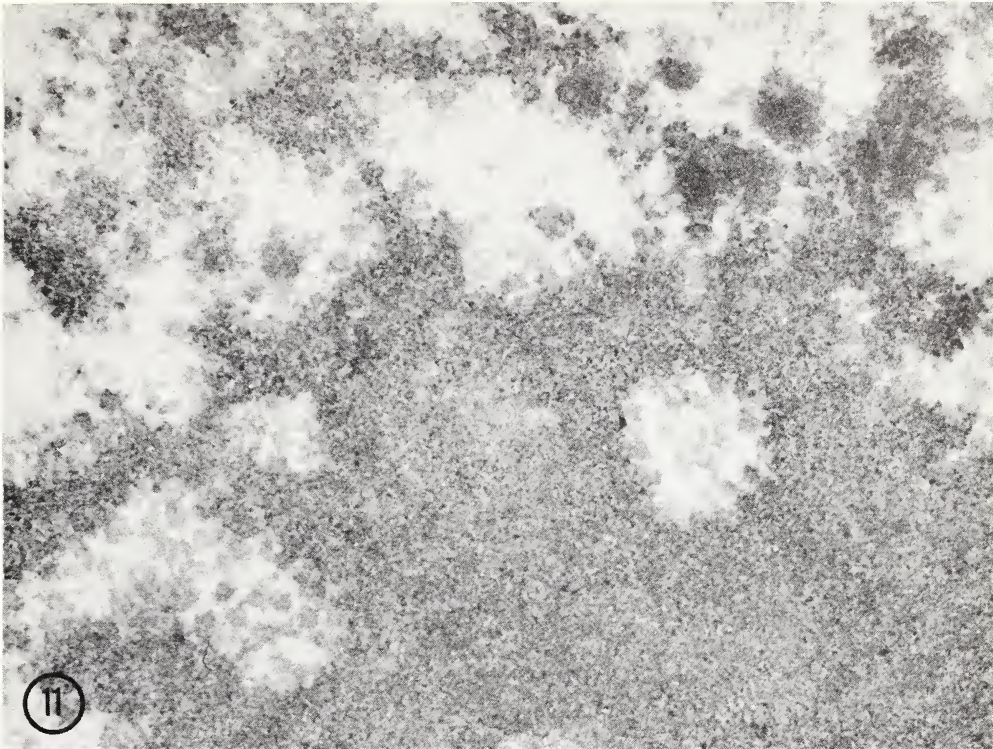
PLATE 30

FIGURE 11.—Enlarged upper left portion of figure 10 shows peripheral structure of abnormal nucleolus (type 6, 6^o) in T6-9b. $\times 81,000$

FIGURE 12.—Complex lamellate basophilic body seen in both normal and abnormal microspores (here in binucleolate nucleus of T6-9b). $\times 31,000$

FIGURE 13.—Structure, similar to that shown in figure 12, from another T6-9b nucleus. $\times 44,500$

FIGURE 14.—Complex granular and filamentous basophilic body, seen in both normal and abnormal microspores, here in a normal nucleus of T6-9a. A portion of the nucleolus is at the *left*. $\times 26,000$



DISCUSSION

Vincent: I would like to ask Dr. Swift how he justifies his conclusion that there is such a distinct difference between the nucleolar bodies found in the presence and absence of the organizer. It would appear that the major difference would be fewer of the small peripheral granules, *i.e.*, a quantitative rather than a qualitative change.

Swift: We were surprised to see how similar in appearance the normal nucleoli of the early microspores were to some of the nucleolus-like bodies in the anucleolate cells. This is accentuated by the fact that the nucleolus-like bodies tend to fuse together with time, so that they may form bodies comparable to true nucleoli in size. However, a little later in microspore development the normal nucleoli become, as we have said, more diffuse at the edge, with the nucleolonemal structure apparent, and there are many particles present at the periphery. At this time the inclusions in anucleolate nuclei remain small and round in outline. At later stages at least some of the anucleolate microspores show signs of involution and necrosis. I would agree with you that, except for the peripheral region of particles, the inclusions in normal and anucleolate cells may appear quite similar. Thus there is apparently nothing about the finely filamentous RNA-containing material, that normally forms the central mass of the nucleolus, that is specific for the organizer locus.

Ritossa: Did you do some cytochemistry or autoradiography of the bodies in the cells without organizers?

Swift: We have not yet made autoradiographs of this material.

Mandel: It appears from Dr. Perry's paper in this Symposium and from our own results (Federation of European Biochem Soc. 2d meeting, 1965, Abstr e79) that there are at least two sites of RNA synthesis in the nucleus. Do you have some evidence from your electron microscopic studies that ribosomes or ribosome-like particles are going from nucleoli to catch messenger RNA (mRNA) on the chromosomes? There is some indirect evidence in bacteria that mRNA is bound to ribosomes as soon as it is formed [Grunberg-Manago and Gros, Bull Soc Chim Biol (Paris) 46: 1441-1497, 1964].

Swift: As I mentioned earlier, the puff particles in *Drosophila* and *Chironomus* are frequently large (400 Å in the Balbiani ring) and quite regular in size, and they apparently contain both RNA and protein. At least some of the RNA, but not the protein, seems to be synthesized at the puff locus itself. We know absolutely nothing else about them, but it is possible that puff particles contain some contribution from ribosomes, forming a complex between messenger RNA and one component of the ribosome, as suggested by McConkey and Hopkins (J Molec Biol 14: 257, 1965) for HeLa cells.

Ritossa: I think that the work of Edström and Beermann (J Cell Biol 14: 371-379, 1962) tends to contradict this view of ribosomes going to catch messenger at the puff level, because they found quite sharp differences in base composition of the RNA from puffs, nucleoli, and cytoplasm.

Swift: I agree that the determinations of Edström and Beermann (J Cell Biol 14: 371, 1962) tend to argue against this hypothesis, but these determinations were made on mixtures of more than one kind of RNA. The variation encountered was not large enough to rule out the possibility that some ribosomal RNA might complex with messenger within the puff.

Anisonucleolinos in Mammalian Cell Cultures^{1,2}

ROBERT LOVE,³ *Department of Pathology, Jefferson Medical College, Philadelphia, Pennsylvania*

SUMMARY

Ribonucleoprotein-containing spherules (nucleolini) in the nucleoli of more than 200 preparations of 10 primary or early passage diploid cell cultures were uniform in size within the same nucleus (isonucleolinos). The nucleolini of several hundred preparations of 26 aneuploid or quasidiploid transformed or neoplastic cell lines varied considerably in size within a given nucleus (anisonucleolinos). These morphological features of the nucleolus were noted during logarithmic and stationary phases of growth and in cells grown in a number of different media. The degree of anisonucleolinos, measured by the proportion of nuclei exhibiting this feature, was greatest in HeLa, RPMI 2650, I407, J111, P147, Pomerat's carcinoma, and KB cells (80–97%) and least in WI8Va 2 cells (15–20%). No simple correlation exists

between the degree of aneuploidy and the percentage of anisonucleolar cells. Conspicuous anisonucleolinos was observed in RPMI 2650 cells which were quasidiploid. Aneuploid micronuclei following treatment of WI38 cells with colchicine were isonucleolar. Anisonucleolinos occurs in cells with an altered genome, as evidenced by aneuploidy, viral infection, or viral-induced transformation. It can be induced by the carcinogen nitroquinoline *N*-oxide or by inhibition of the expression of the genome by actinomycin or fluorodeoxyuridine. The morphology of the nucleolar ribonucleoprotein appears to reflect changes in the structure or function of the genome and may be an indication of the functional activity or genetic expression of euchromatin in the interphase cell.—*Nat Cancer Inst Monogr* 23: 167–180, 1966.

AT LEAST two types of ribonucleoprotein (RNP) have been demonstrated in the nucleolus by the toluidine blue-molybdate method (TBM) (1). One type of RNP is stained metachromatically by TBM, methods A and B, and consists of multiple, small, apparently solid or sometimes hollow spherules for which the classical term nucleolini has been used (2). These structures can also be visualized as minute vacuoles in living cells by phase, or even ordinary light microscopy (3), or by precipitation of

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported by Public Health Service research grant CA-05402-06 from the National Cancer Institute.

³ I wish to thank Oksana Bohachevsky and Richard J. Walsh for their valuable technical assistance.

lead in unfixed cells (4). The other type of RNP is metachromatically stained by TBM, method C, and constitutes the main body of the nucleolus, which appears amorphous in living cells and has therefore been termed the pars amorpha (1, 2). The present report describes a characteristic difference in the morphology of nucleoli of diploid and nondiploid cells *in vitro* and the results of experiments designed to elucidate the mechanisms responsible for this difference.

MATERIALS AND METHODS

Cells.—The various types of cells and the media used in the experiments are summarized in tables 1 and 2.⁴ References to the origin and karyotype of most of the cells have been given in a previous communication (7). The human fetal lung and muscle strains and the strain derived from a testicular teratoma were grown in this laboratory and the karyotype was found to be diploid independently by Dr. Laird Jackson and ourselves (8). Cells were grown in ring cultures (9) in a humidified atmosphere containing approximately 5% CO₂. The rate of growth of cultures was determined by enumeration of cells in an electronic counter (Coulter Electronics).

TABLE 1.—Characteristics of primary and early passage cultures and diploid cell strains exhibiting isonucleolinosi

Cells* and medium	Number of cultures examined	Origin
WI38..... Passages 17, 20†, 23†, 24†, 27, 28, 30, 33.	100	Human fetal lung, female
W18CM..... Passage 22.	2	Human adult buccal mucosa, male
Primary culture.....	4	Mouse fibroblasts
Secondary passage†.....	4	Fetal mouse
Primary culture† A.....	4	Chick embryo fibroblasts
Secondary passage†.....	4	Chick embryo fibroblasts
Secondary passage†.....	4	Calf kidney
JMC#1..... Passages 3, 4, 5, 16, 17, 29†	34	Human fetal lung, male
JMC#2..... Passages 3, 4, 5, 16, 17.	74	Human fetal skeletal muscle, male
JMC#3..... Passage 5.	4	Human testicular teratoma

*All cells were grown in Eagle's basal medium containing 10% calf serum, 2 mM glutamine and antibiotics (penicillin, 100 units/ml, streptomycin 100 µg/ml, kanamycin, 25 µg/ml, or aureomycin, 50 µg/ml), except as indicated by A, where Eagle's minimal essential medium (MEM) and 10% horse serum were substituted, and B, where McCoy's medium (5) was substituted. HeLa cells were grown in basal medium and MEM.

†Preparations coded and recognized as belonging to the above categories by the morphology of the nucleolini.

⁴I should like to thank Drs. V. Defendi, L. Hayflick, A. J. Girardi, M. V. Fernandes, J. Pontén, and F. Jensen of the Wistar Institute and Drs. A. E. Greene and W. Nichols of the South Jersey Medical Foundation for supplying cultures and providing information on the karyotype.

TABLE 2.—Characteristics of aneuploid or quasidiploid cell lines exhibiting anisonucleolinos

Cells	Number of cultures examined	Aniso-nucleo-linosis	Origin
HeLa (3 lines) A.....	100	++++	Human epidermoid carcinoma
RPMI 2650*.....	3	++++	Human epidermoid carcinoma?
HEp-2*.....	6	++	Human epidermoid carcinoma
WISH*.....	4	+++	Human amnion
I407*.....	8	++++	Human intestinal epithelium
Ehrlich ascites.....	50	++++	Mammary tumor, mouse
WI8Va 2*.....	5	+	Human mucosa SV40-trans-formed; infected more than 2 years previously; male
WI38Va 1.....	2	++	Human fetal lung SV40-transformed
J111*.....	16	++++	Human monocytic leukemia
WGM 1*.....	2	++	Green monkey kidney spontaneously transformed
P113.....	4	++	Hamster embryo transformed polyoma
LRKT Passage 124*.....	12	++	Polyoma-induced tumor in rat
P147 #8 Passage 230*.....	8	++++	Hamster, polyoma transformed
4198*.....	6	+++	Mouse, polyoma transformed
89Pt*.....	3	++	Hamster tumor induced by adenotype 12, 1 year previously
W89Va 1.....	2	+	Human, transformed, SV40-infected 4/12 previously
L cells.....	2	++++	Mouse fibroblasts
Pomerat*.....	3	++++	Human carcinoma of colon
L132*.....	8	+++	Human embryonic lung
Dede*.....	6	+++	Chinese hamster lung
W34Va 1.....	2	++	Human adult skin transformed, SV40-infected 6/12 before
KB*.....	8	++++	Human carcinoma of oral cavity
BS-C-1* B.....	6	++	Green monkey kidney
Walker (6)*.....	2	++++	Rat tumor
WI38Va 13 A Passage 131*.....	6	++	Human, SV40-transformed, female
WI26Va 4 Passage 206*...	6	++	Human, SV40-transformed, male

*Preparations coded and recognized as nondiploid by morphology of nucleoli.

Morphology of nucleoli.—At various intervals after seeding, cells were stained by TBM method B, and examined in green light (Kodak Wratten filter #58). Since the optimum concentration of toluidine blue varied slightly with different types of cells, two preparations of each cell type were stained with 0.007 and 0.009% toluidine blue, respectively. (One exception to this was the L cell which required a 0.004% solution of dye for optimal staining. All preparations of toluidine blue certified by the Biological Stain Commission within the last 3 years are satisfactory for the TBM stain.) If the concentration of dye was too high or the cells were degenerating, as in the stationary phase of nondiploid lines, irregular staining of the chromatin occurred. Since irregular staining of nucleolar-associated chromatin might be mistaken for staining of nucleolini, nucleoli in nuclei with irregularly stained chromatin were not scored. In the later stages of the study, this source of error was prevented by treatment of

TABLE 3.—Effects of carcinogen, antineoplastic and colchicine on the nucleolini of WI38 diploid cells

Chemical applied and number of cells	Dosage	Time of exposure	Effect on cells or growth	Effect on nucleolini	Duration of effect
Nitroquinoline N-oxide..... 76 × 10 ³ cells.....	10 ⁻⁴ , 10 ⁻⁵ M..... 10 ⁻⁶ M.....	4 days..... 4 days.....	Kills cells..... Slight inhibition of growth	Disappearance..... Aniso*.....	Permanent At least 1 month
Colchicine..... 99 × 10 ³	2-5 × 10 ⁻⁸ M.....	24 hours.....	Post-C-mitotic micro-nuclei	Iso†, increase or decrease in size	At least 8 days
Actinomycin D..... 67 × 10 ³ cells	0.01 µg per ml.....	3 hours.....	Reversible inhibition of growth	Aniso.....	9 days
Fluorodeoxyuridine..... 67 × 10 ³	8 × 10 ⁵ M.....	48 hours.....	Reversible inhibition of growth	Aniso and disappearance..	96 hours
Canavanine..... 80 × 10 ³	16, 32 and 64 µg per ml	48 hours to 8 days	Inhibition of growth.....	Iso, enlargement.....	At least 8 days

*An iso=anisonucleolinos. † Iso=isonucleolinos.

preparations with deoxyribonuclease [0.001%, Worthington Biochemical in (trishydroxymethyl) aminomethane buffer containing Mg^{++} and Ca^{++} , at pH 7.3 (10) for 2 hours] before staining by TBM, method B. All staining of nucleolini by method B was due to the presence of RNA in them since staining could be prevented by previous digestion with ribonuclease (1, 10).

Experimental procedures designed to elucidate the nature of the nucleolar abnormality in nondiploid cells.—Cultures of diploid WI38 cells were exposed to the carcinogen, nitroquinoline *N*-oxide, the antimetabolic, colchicine, and the antimetabolites actinomycin, 2'-deoxy-5-fluoro-uridine (FUDR), and canavanine sulfate in the concentrations and for the times indicated in table 3. Since it has been shown that the concentration of the cells, as well as that of an antimetabolite, influences the effectiveness of the antimetabolite (11), the cell concentration at the beginning of one experiment with each chemical is also stated in table 3. In all instances, untreated control cultures were stained and examined at the same times as the treated groups.

RESULTS

The size of the spherical nucleolini of cells in primary or early passage cultures and in diploid strains (table 1) varied from cell to cell in each preparation and from one type of culture to another. During logarithmic growth, the nucleolini were larger and more numerous than during the stationary phase of culture. The nucleolini in all the nucleoli of a given cell were almost invariably the same size (isonucleolinosis) and were evenly distributed, or sometimes linearly arranged like streptococci in the body or pars amorphia of the nucleolus (figs. 1, 9, and 11). In some preparations, a slight variation in the size of nucleolini occurred, but this was never found in more than 6% and rarely in more than 3% of the nuclei of a diploid culture. On the other hand, in all 26 nondiploid cultures examined (table 2) the nucleolini in a considerable proportion of nuclei varied markedly in size in the same nucleolus and in different nucleoli within the same nucleus (anisonucleolinosis). The degree of anisonucleolinosis, as measured by the proportion of nuclei exhibiting this phenomenon, was variable (+ to 4+, table 2), being least in W18Va 2 cells (approximately 15–20%) and greatest in HeLa, RPMI, 2650, I407, J111, P147, Pomerat's carcinoma, KB, Ehrlich, and the Walker tumor cells (80–99%). The difference between the diploid and the nondiploid cells was such that each type could be readily distinguished by the author (*see* daggers in table 1 and asterisks in table 2) and by a number of scientists unfamiliar with the appearance of these organelles. The appearance of the nucleolini in a number of different cells is illustrated in figures 1–12. Not uncommonly, there was one very large nucleolus and many small ones in an anisonucleolar cell (fig. 8).

The effects of various chemicals on the isonucleolar nucleoli of WI38 cells are summarized in table 3. High concentrations of nitroquinoline *N*-oxide killed the cells but 10^{-6} M solutions resulted in slight inhibition of growth (12% in 24 hours) and produced approximately 70% anisonucleolar cells in 24 hours (fig. 13). The cells gradually ceased to multiply but remained anisonucleolar during the longest period of observation to date (1 month). Classical C-mitosis was produced by colchicine $2-5 \times 10^{-8}$ M). Multiple irregularly sized micronuclei formed after removal of the colchicine. The nucleoli in the micronuclei were smaller than those in mononucleate cells but remained isonucleolar. The nucleoli of the mononucleate cells were enlarged as compared with those of control untreated cells. This feature was utilized to produce enlargement of small nucleoli to obtain clearer microphotographs (fig. 9). Treatment with actinomycin D for 3 hours resulted in anisonucleoliosis of 70-80% of cells, apparently as a result of irregular fusion of preexisting nucleoli to produce larger ones of uneven size (table 3 and fig. 14). Treatment with FUDR resulted in irregular loss of stainability of the nucleoli and, after 48 hours, RNA was no longer stainable in the nucleoli. Forty-eight hours after FUDR was removed, the nucleoli were again isonucleolar. Canavanine sulfate in concentrations of 16-64 μ g per ml resulted in some inhibition of growth and enlargement of nucleoli which remained isonucleolar (table 3).

DISCUSSION

For many years cytologists and pathologists have noted differences between the nucleoli of normal and neoplastic cells, and the presence of enlarged or bizarre nucleoli has been one of the criteria in the diagnosis of malignant tumors. Overemphasis of these differences has prompted Willis to warn that: "Others have become obsessed with nuclear and nucleolar changes as a means of recognizing tumor cells." (12). After an extensive investigation of the problem, Cowdry and Paletta (13) concluded that "no single cellular measurement made yielded results so characteristic of malignant cells as to differentiate them invariably from neighboring hyperplastic epidermal cells." More recently the attention of cytologists has turned toward the internal structure of the nucleolus (14), but again, no characteristic feature distinguishes all normal from all neoplastic cells.

The present report describes differences between the nucleoli of diploid cells and those of aneuploid or quasidiploid established cell lines. Certainly, 9 out of the 10 diploid strains were derived from normal tissue and, quite probably, the cells grown from the teratoma may also be of stromal or non-neoplastic origin. All the anisonucleolar cells have become morphologically transformed spontaneously or by oncogenic viruses, or they were grown from malignant neoplasms. Such cultures cannot, however, be compared directly with spontaneous tumors *in vivo* and must

have undergone extensive progression (15) during repeated passage *in vitro*. It is unfortunately impossible, at present, to study the size of nucleolini in sections of spontaneous tumors for technical reasons. The nucleolini are embedded at different levels in the substance of the nucleoli so that sections would be bound to cut through them in different planes, resulting in artifactual differences in size. It must also be emphasized that the difference between diploid and nondiploid cells is not clear cut. Although the modal number of chromosomes of "diploid" cell cultures remains diploid throughout their life span, increasing numbers of aneuploid cells appear in the later passages (16). At the present time, perhaps the only unique difference between anisonucleolar and isonucleolar cells is that the latter appear to have a limited life span (17). On the basis that the difference between diploid isonucleolar and nondiploid anisonucleolar cells might be a neoplastic transformation, the effects of the carcinogen nitroquinoline *N*-oxide on diploid cells were studied. With appropriate doses, a large proportion of WI38 cells became anisonucleolar and survived for a month. It is hoped to be able to grow these cells to study the karyotype and other aspects of their biological behavior.

No simple correlation appears to exist between the degree of aneuploidy and the percentage of anisonucleolar cells. The RPMI 2650 tumor was particularly notable in this respect, since it exhibited maximal anisonucleolinos and was quasidiploid with only one abnormal chromosome. The effects of colchicine lend further support to the lack of correlation between chromosome ploidy and anisonucleolinos. The isonucleolar micronuclei that form after C-mitosis were very variable in size and must have contained variable numbers of chromosomes. It is probable, however, that the post C-mitotic cells, as distinct from the micronuclei, were not aneuploid but tetraploid, and therefore euploid.

The absence of a direct relationship between chromosome number and nucleolar morphology does not, however, imply an absence of genetic control over nucleolar structure and function. Inhibition of DNA-primed synthesis of RNA by actinomycin (11) produced anisonucleolinos. Inhibition of synthesis of DNA by FUDR resulted in inhibition of nuclear synthesis of RNA (11) and produced anisonucleolinos. Following removal of the inhibitor, the cells reverted to the isonucleolar state (table 3). It was hoped that by inhibiting synthesis of arginine by canavanine (18) it might be possible to influence histone metabolism and thereby depress some aspect of genetic function which might be revealed by anisonucleolinos. Failure to do this may have been due to the fact that the effect of the antimetabolite was reversed by the arginine in the medium. Inhibition of growth indicated, however, that the antimetabolite did have some effect. Other evidence for genetic control of nucleolar RNA comes from studies of the effects of viral infection on cells. Infection of a number of cells by different cells by deoxyriboviruses, such as polyoma, herpes (19), and SV40 (20) virus, results in striking changes in the nucleolini. The only one of these viruses that was studied in diploid cells, namely,

SV40, produced anisonucleolinos in the acute stage of infection (20) and, at a later stage in the transformed cells, presumably after incorporation of viral genetic material into the cell genome.

In conclusion, anisonucleolinos occurs in cells with an altered genome as evidenced by aneuploidy or by viral infection or viral-induced transformation. It can be induced by a carcinogen or by inhibition of the expression of the genome by actinomycin and FUDR. The morphology of the nucleolini may reflect changes in the structure or function of the genome and may be an indication of the functional activity or genetic expression of euchromatin in the interphase cell.

RESUMEN

Las esférulas que contienen ribonucleoproteína (nucleolinos) en los nucleolos de más de 200 preparaciones de diez pasajes tempranos primarios o cultivos de células diploides fueron de tamaño uniforme dentro del mismo núcleo (isonucleolinos). Los nucleolinos de varios cientos de preparaciones de 26 líneas celulares aneuploides, o transformadas cuasidiploides o neoplásicas, variaron considerablemente en tamaño dentro de un núcleo dado (anisonucleolinos). Se notaron estas características morfológicas de los nucleolos durante las fases estacionarias y de crecimiento logarítmico y en las células cultivadas en diferentes medios. El grado de anisonucleolinos, medido mediante la proporción de núcleos que exhiben este rasgo fue mayor en HeLa, RPMI 2650, I407, J111, P147, carcinoma de Pomerat y células KB (80-97 %) y al menos en células WI8Va 2 (15-20 %). No existe una correlación simple entre el grado de aneuploidía y el porcentaje de células anisonucleolinales. Se observó una notoria anisonucleolinos en células RPMI 2650 que eran cuasidiploides. Los micronúcleos aneuploides que resultan del tratamiento de las células WI38 con colchicina fueron isonucleolinales. La anisonucleolinos se produce en células con genotipo alterado, como evidencia de aneuploidía, de infección por virus o transformación inducida por virus. Puede inducirse por el carcinógeno óxido-N de nitroquinolina o por inhibición de la expresión del genotipo por actinomicina o fluorodeoxiuridina. La morfología de la PRN nucleolar parece reflejar cambios en la estructura o función del genotipo y puede ser una indicación de la actividad funcional o la expresión genética de la eucromatina en la célula interfásica.

REFERENCES

- (1) LOVE, R., and WALSH, R. J.: Studies of the cytochemistry of nucleoproteins. II. Improved staining methods with toluidine blue and ammonium molybdate. *J Histochem Cytochem* 11: 188-196, 1963.
- (2) LOVE, R., and BHARADWAJ, T. P.: Two types of ribonucleoproteins in the nucleolus of mammalian cells. *Nature (London)* 183: 1453-1454, 1959.
- (3) BALBIANI, E. G.: Sur les mouvements qui se manifestent dans la tache germinative chez quelques animaux. *CR Soc Biol (Paris)* 1: 64-68, 1864.
- (4) STUDZINSKI, G. P., and LOVE, R.: Nucleolar organelles shown by lead precipitation in unfixed cultured cells. *Stain Tech* 39: 397-401, 1964.
- (5) MCCOY, T. A., MAXWELL, M., and KRUSE, P. F.: Amino acid requirements of the Novikoff hepatoma *in vitro*. *Proc Soc Exp Biol Med* 100: 115-118, 1959.
- (6) WRBA, H., EICKE, J., and MEINERS, M. L.: Veränderungen im chromosomensatz heterolog wachsender tumoren. *Z Krebsforsch* 66: 115-118, 1964.

- (7) LOVE, R.: Differences in the internal structure of nucleoli of diploid and non-diploid transformed or neoplastic cells *in vitro*. *Exp Cell Res* 40: 188-192, 1965.
- (8) MOORHEAD, P. S., NOWELL, P. C., MELLMAN, W. J., BATTIPS, D. M., and HUNGERFORD, D. A.: Chromosome preparations of leukocytes cultured from human peripheral blood. *Exp Cell Res* 20: 613-616, 1960.
- (9) WILDY, P., SMITH, C. L., NEWTON, A. A., and DENDY, P.: Quantitative cytological studies on HeLa cells infected with herpes virus. *Virology* 15: 486-500, 1961.
- (10) LOVE, R., and RABOTTI, G.: Studies of the cytochemistry of nucleoproteins. III. Demonstration of deoxyribonucleic acid-ribonucleic acid complexes in mammalian cells. *J Histochem Cytochem* 11: 603-612, 1963.
- (11) LOVE, R., STUDZINSKI, G. P., and ELLEM, K. A. O.: Use of antimetabolites in the study of normal and abnormal cell structure and function. *Fed Proc* 24: 1206-1216, 1965.
- (12) WILLIS, R. A.: Pathology of Tumours, 3d ed. Washington, Butterworths, 1960, p 132.
- (13) COWDRY, E. V., and PALETTA, F. X.: Changes in cellular, nuclear, and nucleolar sizes during methycholanthrene epidermal carcinogenesis. *J Nat Cancer Inst* 1: 745-759, 1941.
- (14) BERNHARD, W., and GRANBOULAN, N.: The fine structure of the cancer cell nucleus. *Exp Cell Res Suppl* 9: 19-53, 1963.
- (15) FOULDS, L.: Tumor progression. *Cancer Res* 17: 355-356, 1957.
- (16) SAKSELA, E., and MOORHEAD, P. S.: Aneuploidy in the degenerative phase of serial cultivation of human cell strains. *Proc Nat Acad Sci USA* 50: 390-395, 1963.
- (17) HAYFLICK, L.: The limited *in vitro* lifetime of human diploid cell strains. *Exp Cell Res* 37: 614-636, 1965.
- (18) ACKERMANN, W. W., COX, D. C., and DINKA, S.: Control of histone and DNA synthesis with canavanine, puromycin and poliovirus. *Biochem Biophys Res Commun* 19: 745-750, 1965.
- (19) LOVE, R., RABSON, A. S., and WILDY, P.: Changes in the nucleolus of normal and neoplastic cells infected with riboviruses and deoxyriboviruses. *Acta Un Int Cancer* 20: 1384-1387, 1964.
- (20) LOVE, R., and FERNANDES, M. V.: Cytological and cytochemical studies of green monkey kidney cells infected *in vitro* with simian virus 40. *J Cell Biol* 25: 529-543, 1965.

PLATE 31

FIGURE 1.—Nucleolus of WI38 cell. The nucleolini are all the same size and are evenly scattered throughout the body or pars amorpha of the nucleolus. Some of the nucleolini appear to be in short chains like streptococci. All preparations stained by toluidine blue-molybdate, method B. $\times 2,800$

FIGURE 2.—Nucleolus of a BSC-1 transformed green monkey kidney cell. The nucleolini vary greatly in size and some of the larger ones appear to have unstained centers (*arrows*). $\times 2,800$

FIGURE 3.—Nucleus of an I407 intestinal epithelial cell containing two nucleoli within which the nucleolini vary greatly in size. $\times 2,800$

FIGURE 4.—Nucleus of a J111 human monocytic leukemia cell. At least four nucleoli exhibit striking anisonucleolinos. $\times 2,800$

FIGURE 5.—Marked anisonucleolinos of a nucleolus of a HEp-2 cell. $\times 2,800$

FIGURE 6.—Two anisonucleolar nucleoli in a HeLa cell. $\times 2,800$

FIGURE 7.—An isonucleolar nucleolus in a SV40-transformed culture (W34Va 1). $\times 1,400$

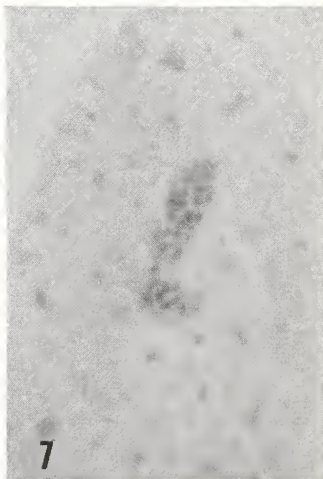
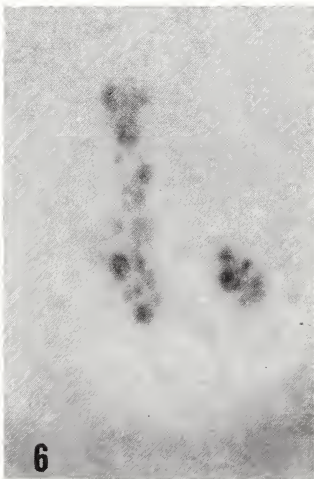
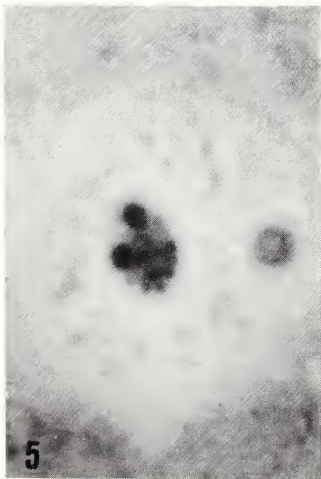
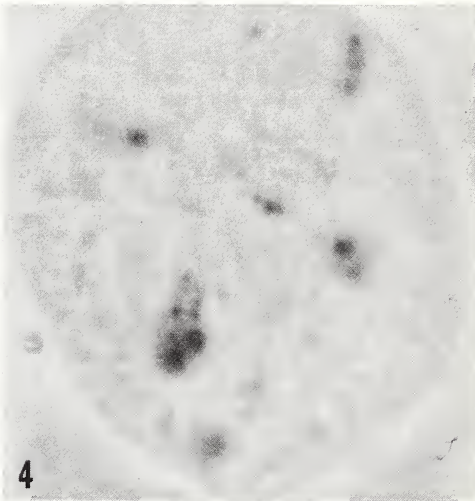
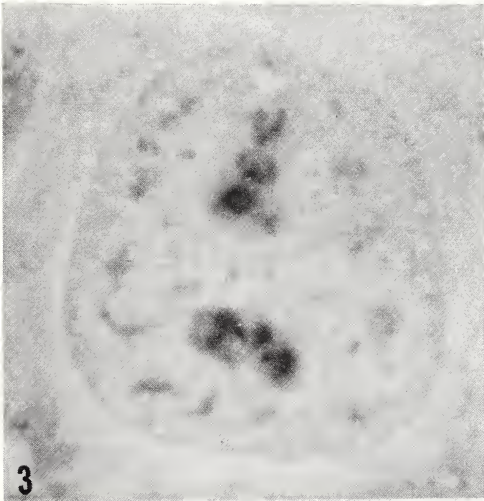
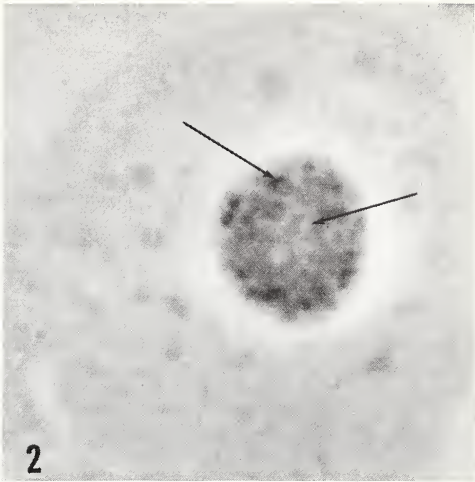
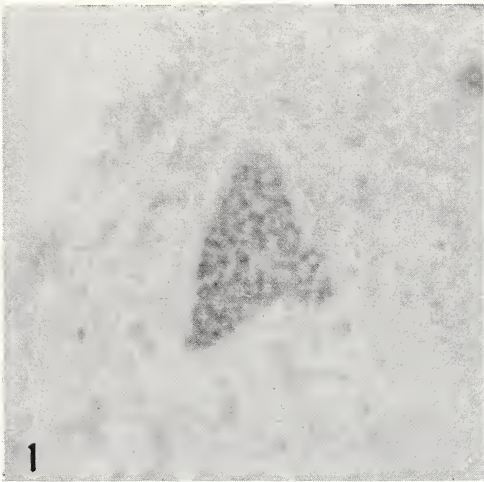


PLATE 32

FIGURE 8.—One large nucleolus and many small ones in a nucleus of a HeLa cell.
× 2,800

FIGURE 9.—Two isonucleolar nucleoli in WI3S cell treated with colchicine (5×10^{-8} M). × 2,800

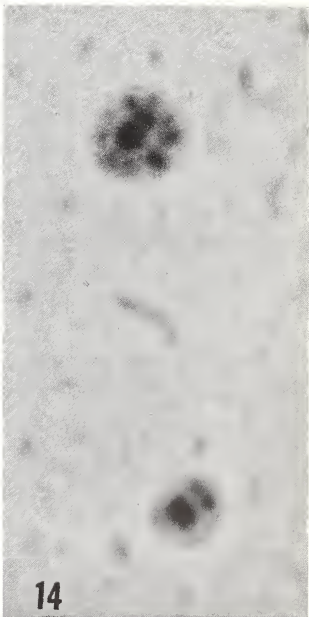
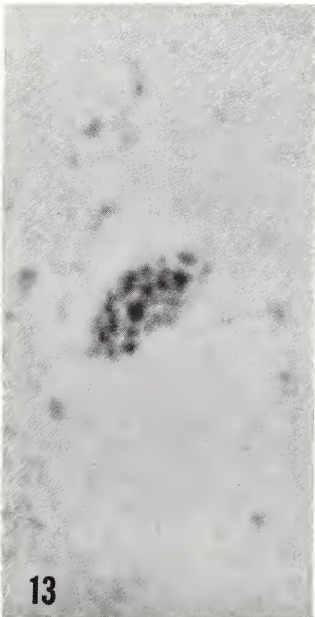
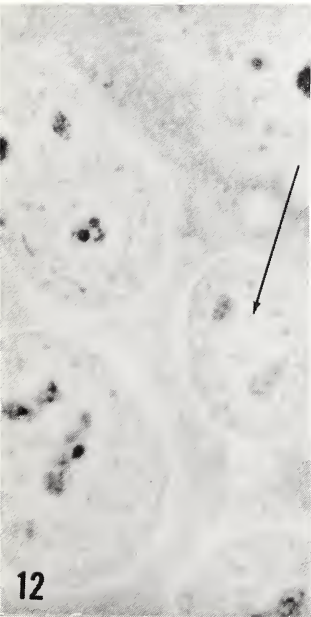
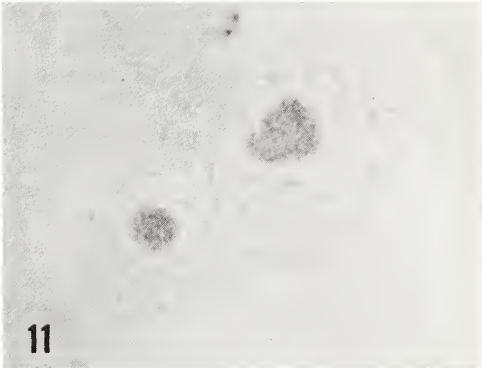
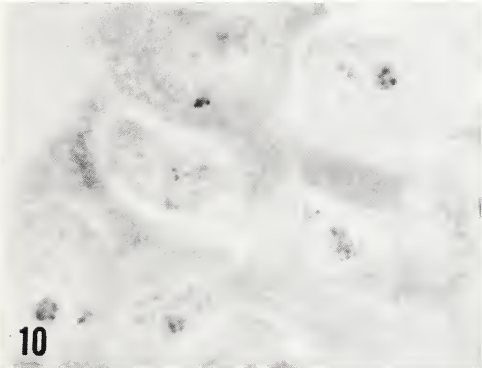
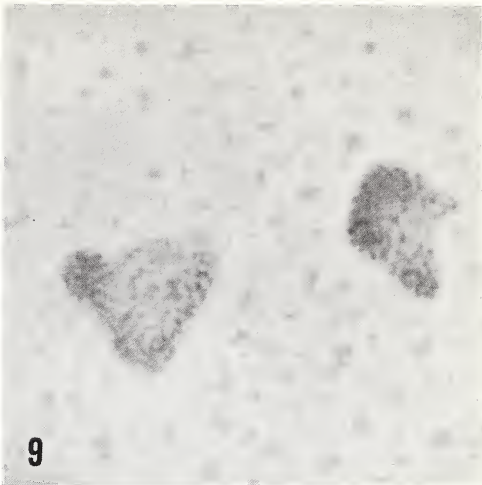
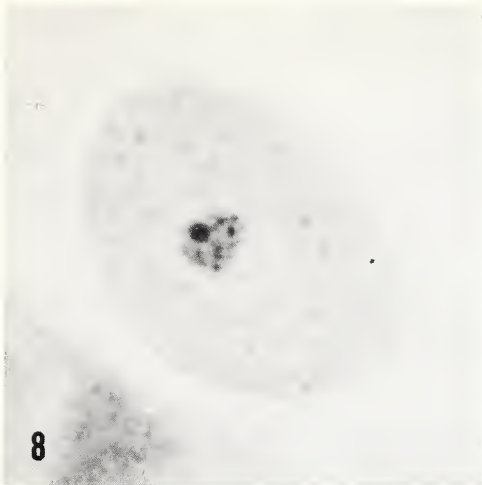
FIGURE 10.—Several anisonucleolar cells from Pomerat's carcinoma. × 1,400

FIGURE 11.—Two isonucleolar nucleoli in a nucleus of a W1SCM cell. × 1,400

FIGURE 12.—One isonucleolar (*arrow*) nucleus and several anisonucleolar nuclei from a HEp-2 culture. × 1,400

FIGURE 13.—Anisonucleolinosi induced in a WI3S cell by nitroquinoline N-oxide.
× 3,500

FIGURE 14.—Anisonucleolinosi of a WI3S cell induced by actinomycin. × 4,000



DISCUSSION

Perry: A point of clarification: Did you say that all normal, nontumorous tissue when put in culture exhibits isonucleolinoses or were the normals that you showed sections from whole tissue? I want to know if this has anything to do with putting the cells in culture. Are primary cultures of nontumorous tissue *aniso* or *iso*?

Love: All the primary cultures that we have grown, such as chicken embryo fibroblasts or mouse fibroblasts, are *iso*. All the observations were made on tissue cultures.

Estable: First, I wish to ask you in what sense do you use the word nucleolini, because it seems that you give the same name to many different structures and forms? Second, are these similar or homologous to the paranucleoli of Cajal (Ramon y Cajal, Trabajos del Laboratorio de Investigaciones Biologicas de la Univ de Madrid 8: 27-62, 1910)?

Love: The diploid cells have no more than one or two in about 90 percent of the cells, so I think they are almost certainly nucleoli.

Kasten: Perhaps the question that was just raised relates to the terminology used. As I understand your use of the term, nucleolus, this refers to a spherule. Is this structure identical to any of the structures described earlier, namely, the nucleolonema or pars amorpha? What are the actual morphological relationships?

Love: I think I can save a lot of time by saying I wish I knew.

Sirlin: I tried to find this correlation in your previous papers and I came to the conclusion that your bodies are generally near vacuoles. The nucleolini are around vacuoles, whereas the nucleolonema is distributed differently. Is that right?

Love: I think that it is a little more complicated. I agree that in the living cell the nucleolini appear as vacuoles. However, we also see vacuoles in cells in addition to nucleolini, so that there are at least two kinds of vacuoles.

Busch: It is very important that one establish whether the nucleolini represent a difference in composition of ribonucleoprotein or whether it is simply a difference in states of aggregation.

Love: I think that the work that you are doing with isolated nucleoli may help to answer this. We have some evidence that the two types of ribonucleoprotein in the nucleolus are functionally different. Treatment of HeLa cells with fluorodeoxyuridine leads to loss of RNA in the nucleolini without affecting the other RNA of the nucleolus. I envisage the nucleolini as something comparable to Balbiani rings or puffs and their morphology probably reflects the function of the genome during interphase.

Detection and Origin of Nucleolar Components: A Model for Nucleolar RNA Function¹

C. J. TANDLER,² *Instituto Fitotécnico de Santa Catalina, Llavallol y Instituto de Anatomía General y Embriología, Facultad de Ciencias Médicas, Buenos Aires, Argentina*

SUMMARY

Cytochemical techniques indicated that, besides RNA, two other major components—a large pool of orthophosphate ions and a firmly bound, non-RNA silver-reducing substance—were sharply localized in the nucleolus. These two components were absent from metaphase chromosomes. The nucleolus was initiated during telophase by the formation of silver-reducing material along all chromosomes in places other than the locus of the nucleolar organizer. This material fused into small bodies which gradually collected at the organizer. With autoradiography, differences have

been found in nucleolar RNA by using different labeled precursors. A model for the interrelationship of the RNAs at the nucleolus which takes into account the available cytological and biochemical data is presented. While the nucleolar organizer locus primes for the ribosomal RNA found in the particles of the granular zones, other loci in all chromosomes contributed to the RNA of the fibrillar zone and the dense, silver-reducing, protein matrix. The nature of the RNA in the fibrillar zones is discussed.—*Nat Cancer Inst Monogr* 23: 181–190, 1966.

WITH CYTOCHEMICAL techniques I have shown that, besides RNA, two other major components are sharply localized in the nucleolus of plant and animal cells; they are absent from metaphase chromosomes. The first is a large pool of orthophosphate ions (figs. 1 and 3), shown by lead acetate precipitation of unfixed cells (1–5). This reaction is completely abolished by any other form of fixation or by heating (fig. 2). The heavy nucleolar precipitate was identified as lead orthophosphate by the *in situ* properties of the lead precipitate as well as by chromatography of lead-fixed tissues (1–4). It is not an artifact, since by inducing diffusion, the phosphate ions do not move to the nucleolus (5). It suggests a “bound” form of this ion since the aqueous phase of organic solvents when added to the lead fixative causes rapid leakage of phosphate from

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

the nucleolus, whereas other substances do not (5). A similar technique has been described more recently by Studzinski and Love (6).

The other substance, which is probably a specific nucleolar substance, was identified by the strong reduction of ammoniacal silver solutions (7) and photographic emulsions in darkness (8). This last property also was discovered independently by Das and Alfert (9) and Das (10). It is not a nucleic acid and is also different from the lead-precipitating component since the reducing property is unaffected by hot trichloroacetic extraction of fixed tissues (7-10). In metaphase this substance is absent from the chromosomes, having been lost to the cytoplasm. The origin of the nucleolus in the daughter nuclei is initiated, during telophase, by the formation of material having the *same* silver-reducing property of the interphase nucleolus and in contact with all chromosomes (figs. 4 and 5). This material fuses into several small bodies—the prenucleolar bodies—which gradually form larger masses (fig. 4) and finally collect at the organizer site to form the definite nucleoli. These bodies also contain RNA (10-12). Therefore, the nucleolus does not originate exclusively from the organizer region of the chromosomes. Silver reduction is not a chemically specific test (7, 8), but identical behavior was demonstrated by Beck (13, 14) with a nucleolar antigen using the fluorescent antibody technique. The silver-reducing substance and rapidly labeled RNA were demonstrated in the nucleolus (or nucleolar-like bodies) of micronuclei lacking the nucleolar organizer (12). In the salivary glands of *Smittia*, where the nucleolus and the nucleolar organizer chromatin can be easily recognized, the silver-reducing substance is delimited to the nucleolus proper (15).

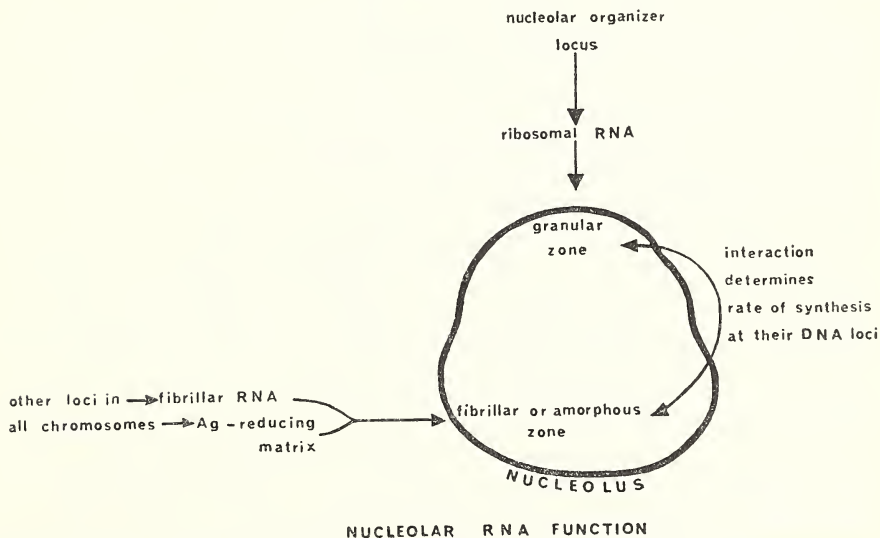
Using autoradiography we have found some striking differences in the patterns of incorporation of RNA with different labeled precursors. In root-tip cells after 1-hour incubation, about twice as much labeled RNA was found in the nucleolus, as compared to the nucleus and cytoplasm, with cytidine as with uridine. Both nucleosides had the tritium in the 5 position of the pyrimidine ring (16). The tritium of uridine goes into both uracil and cytosine bases of RNA as shown by chromatography of alkaline hydrolysates; a lower radioactive uracil/cytosine ratio was indicated for the RNA in the nucleus and nucleolus than in the cytoplasm (17). The situation was similar in rat liver tissue (18). These differences were reflected in pool sizes and metabolic pathways. In tissue culture cells it has been also reported that the nucleolus incorporated more than twice as much cytidine as adenosine (19).

We have also shown that in growing root-tip cells P^{32} -orthophosphate uptake into nucleolar RNA was the lowest in the cell, even with roots grown in P^{32} for one to two mean generation times (fig. 6). This is strikingly different from results with labeled ribonucleosides (20-22) and from the pattern given by P^{32} in Dipteran salivary glands (23). Other dividing cells, such as chick embryo explants and fibroblasts, behave similarly. This indicates that, in these cells, there is no massive contribution of chromosomal RNA to the interphase nucleolus. Under the conditions

used, P^{32} allows 2 to 3 μ resolution (21) so that label in these large nucleoli would be recognized if present, as in the case of salivary glands. Of course, P^{32} could be incorporated into nucleolar RNA below the level of detection with these autoradiographs, which were briefly exposed. Autoradiograms obtained with lead-acetate-fixed root tips incubated with P^{32} also suggested that the large pool of orthophosphate in the nucleolus serves as precursor for nucleolar RNA phosphorus (21).

NUCLEOLAR RNA FUNCTION

I would like to present a model (text-fig. 1) for the interrelationship of the RNAs of the nucleolus which takes into account recent observations at the molecular and electron microscope level. This model may help to resolve some contradictions found in the literature on this problem. The electron microscope reveals that the nucleolus is formed of a granular zone, rich in ribosome-like particles, and a fibrillar or amorphous zone embedded in a dense protein matrix (24). Both zones contain RNA (25-29). It now appears evident that these two nucleolar RNA pools are different and have different origins. This model suggests that the RNA in the granular zone is formed at the nucleolus organizer locus and is ribosomal since Ritossa and Spiegelman (30) have demonstrated that this region in *Drosophila* is complementary to both 18S and 28S ribosomal components. This has an analogy in the case of bacteria, which lack nucleoli but have the cistrons for ribosomal components clustered in a discrete portion of the chromosome (31). On the other hand, the RNA found in the dense fibril-



TEXT-FIGURE 1.—Proposed model for the functioning and origin of the two RNA-containing nucleolar constituents.

lar zone is not synthesized at the organizer locus, since Jones (32) and others (this Symposium) have shown recently that this is the only structural component found in the nucleolus-like bodies of the anucleolate mutant of *Xenopus*, the granular component being absent. These spherical bodies appear to contain RNA (33) and are structurally similar to the fibrillar regions of normal nucleoli. Brown and Gurdon (34) have shown that no synthesis of ribosomal RNA or ribosomal RNA precursor molecules takes place in the anucleolate mutant. The RNA in the fibrillar zone cannot be the precursor of ribosomal RNA as has been suggested (27), as it has been shown in annealing experiments that negligible complexing occurs between ribosomal RNA and the anucleolate DNA, *i.e.*, that the mutation involved the loss of most of the ribosomal cistrons (35). Therefore, although the nucleolar pool of ribosomal RNA is primed exclusively at the organizer locus, the RNA of the fibrillar zone (which will be referred to as "fibrillar" RNA) does not appear to be. An impressive body of evidence clearly demonstrates the potentiality for formation of RNA-containing, dense nucleolar-like bodies along all chromosomes in places other than the locus of the nucleolar organizer (33, 36, 37). We suggest that the fibrillar RNA component, together with the dense matrix, is formed at these extra-organizer sites. Other examples which seem to be similar are a *Chironomus* hybrid (38), maize pollen tetrads (39), and also micronuclei lacking nucleolar organizers (12, 40), as well as during the normal process of mitosis and meiosis (41-44). This fibrillar RNA component appears at many points within the chromosome bands in Sciarids which have multiple nucleoli (45), in the RNA-containing spherical bodies found by Lafontaine (46) in meristematic plant cell nuclei, and along the telophase chromosomes where the prenucleolar bodies are located in broad bean root tips (47) (fig. 4). These bodies also share the silver-reducing component of definitive nucleoli (8, 9). Das (12) has demonstrated that all chromosome fragments induced by X rays at telophase and all micronuclei at early interphase contain the silver-reducing substance and that only those micronuclei capable of maintaining this nucleolar component are also active in nucleic acid synthesis.

Once the definitive nucleolus emerges as a collected body, interaction between the two RNA-containing zones determines their rate of synthesis at their chromosomal loci. The nature of this interaction is only speculative at present. The concept of "latent organizers" has been proposed in the past (33, 36, 37, 48), *i.e.*, that the nucleolar organizer collects material from and limits its synthesis by other chromosomal loci. The latent organizer concept is envisaged here as the capacity of all chromosomes to produce the fibrillar RNA associated with the dense amorphous substance.

An apparent "independence" of synthesis is valid for the RNA in both the granular (49) and the fibrillar zones (29). The dual origin of nucleolar RNAs also explains some apparent contradictions found with autoradiography which indicated for different materials that the initial site of RNA labeling is (27, 50) or is not (15, 29, 51, 52) in the chromatin in

close contact to the nucleolus where the organizer is located. This will depend on the amount, rate of synthesis, and morphological distribution of the different constituents of the nucleolar region.

The nature of the fibrillar RNA is unknown at present. RNA of the messenger type is suggestive for fibrillar RNA as it is a rapidly labeled RNA, when present in the nucleolar bodies, and produced at many sites in all chromosomes. In this aspect the nucleolus is envisaged as a mosaic of gene products which interact with the ribosomal cistrons of the organizer locus.

RESUMEN

Las técnicas citoquímicas indican que, aparte del ARN, otros dos componentes principales—un gran reservorio de iones ortofosfato y una sustancia no ARN reductora de la plata—se localizan claramente en el nucleolo. Estos dos componentes están ausentes de los cromosomas metafásicos. El nucleolo se origina durante la telofase por la formación del material reductor de la plata a lo largo de los cromosomas en otros lugares fuera del organizador nucleolar. Este se fusiona en pequeños cuerpos que se acumulan gradualmente en el organizador. Se han hallado, con radioautografía, algunas diferencias notables del ARN nucleolar empleando diferentes precursores marcados. Se presenta un modelo de las interrelaciones de los ARN en el nucleolo que explica los datos citológicos y bioquímicos disponibles. Mientras que el locus del organizador nucleolar prepara el ARN ribosómico encontrado en las partículas ribosómicas de las zonas granulares, los otros loci de los cromosomas forman el ARN de la zona fibrilar junto con la densa matriz proteica reductora de la plata. Se discute la naturaleza del ARN de las zonas fibrilares.

REFERENCES

- (1) TANDLER, C. J.: An acid-soluble component of the nucleolus; the cytochemical specificity of the lead acetate reaction. *J Histochem Cytochem* 4: 331-340, 1956.
- (2) ———: A chemically specific technique for the intracellular localization of inorganic phosphate. *J Histochem Cytochem* 5: 489-499, 1957.
- (3) ———: The localization of inorganic phosphate in the oocytes of *Bufo arenarum*. Heterogeneity of the nucleoli. *Exp Cell Res* 14: 408-413, 1958.
- (4) ———: The localization of intracellular orthophosphate. The role of the nucleoli. *Biochim Biophys Acta* 44: 536-542, 1960.
- (5) ———: The detection of orthophosphate within the cell nucleus. *Exp Cell Res* 25: 246-250, 1961.
- (6) STUDZINSKI, G. P., and LOVE, R.: Nucleolar organelles shown by lead precipitation in unfixed cultured cells. *Stain Techn* 39: 397-401, 1964.
- (7) TANDLER, C. J.: The reaction of nucleoli with ammoniacal silver nitrate in darkness; additional data. *J Histochem Cytochem* 3: 196-202, 1955.
- (8) ———: The silver-reducing property of the nucleolus and the formation of prenucleolar material during mitosis. *Exp Cell Res* 17: 560-564, 1959.
- (9) DAS, N. K., and ALFEET, M.: Detection of a nucleolar component and its behaviour during the mitotic cycle. *Anat Rec* 134: 548-549, 1959.
- (10) DAS, N. K.: Demonstration of a non-RNA nucleolar fraction by silver staining. *Exp Cell Res* 26: 428-431, 1962.

- (11) KLEINFELD, R. G., and VON HAAM, E.: Effect of thioacetamide on rat liver regeneration. II. Nuclear RNA in mitosis. *J Biophys Biochem Cytol* 6: 393-398, 1959.
- (12) DAS, N. K.: Synthetic capacities of chromosome fragments correlated with their ability to maintain nucleolar material. *J Cell Biol* 15: 121-130, 1962.
- (13) BECK, S. J.: The behaviour of certain nuclear antigens in mitosis. *Exp. Cell Res* 28: 406-418, 1962.
- (14) BECK, S. J., ANDERSON, J. R., McELHINNEY, A. J., and ROWELL, N. R.: Anti-nucleolar antibodies. *Lancet* 2: 575-577, 1962.
- (15) SIRLIN, J. L., TANDLER, C. J., and JACOB, J.: The relationship between the nucleolus organizer and nucleolar RNA. *Exp Cell Res* 31: 611-615, 1963.
- (16) TANDLER, C. J., and LA TORRE, J. L.: Differential incorporation of 5'-tritiated pyrimidines into nucleolar RNA of developing root tip cells. *Exp Cell Res* 41: 329-337, 1966.
- (17) ———: The removal of uracil residues from RNA in tissue preparations. *Life Sci* 3: 1431-1435, 1964.
- (18) HECHT, L. I., and POTTER, V. R.: Nucleic acid metabolism in regenerating rat liver. V. Comparison of results *in vivo* and in tissue slices. *Cancer Res* 18: 186-192, 1958.
- (19) PERRY, R. P., ERRERA, M., HELL, A., and DURWALD, H.: Kinetics of nucleoside incorporation into nuclear and cytoplasmic RNA. *J Biophys Biochem Cytol* 11: 1-13, 1961.
- (20) TANDLER, C. J., and SIRLIN, J. L.: Some observations on the nuclear pool of ribonucleic acid phosphorus. *Biochem Biophys Acta* 55: 228-230, 1962.
- (21) ———: Differential uptake of orthophosphate and ribonucleosides into nucleolar ribonucleic acid. *Biochem Biophys Acta* 80: 315-324, 1964.
- (22) WOODS, P. S.: RNA in nuclear-cytoplasmic interaction. *Brookhaven Sympos Biol* 12: 153-174, 1959.
- (23) McMASTER-KAYE, R.: The metabolic characteristics of nucleolar, chromosomal and cytoplasmic ribonucleic acid of *Drosophila* salivary glands. *J Biophys Biochem Cytol* 8: 365-378, 1960.
- (24) MARINOZZI, V.: Cytochimie ultrastructurale du nucléole-RNA et protéines intranucléolaires. *J Ultrastruct Res* 10: 433-456, 1964.
- (25) MARINOZZI, V., and BERNHARD, W.: Présence dans le nucléole de deux types de ribonucléoprotéines morphologiquement distinctes. *Exp Cell Res* 32: 595-598, 1963.
- (26) HYDE, B. B., SANKARANARAYANAN, K., and BIRNSTIEL, M. L.: Observations on fine structure in pea nucleoli *in situ* and isolated. *J Ultrastruct Res* 12: 652-667, 1965.
- (27) GRANBOULAN, N., and GRANBOULAN, P.: Cytochimie ultrastructurale du nucléole, II. Etude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp Cell Res* 38: 604-619, 1965.
- (28) SWIFT, H.: Nucleic acids and cell morphology in Dipteran salivary glands. *In The Molecular Control of Cellular Activity* (Allen, J. M., ed.). New York, Toronto, and London, McGraw-Hill Book Co., 1962, pp 73-125.
- (29) KARASAKI, S.: Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J Cell Biol* 26: 937-958, 1965.
- (30) RTOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (31) DUBNAU, D., SMITH, I., and MARMUR, J.: Gene conservation in bacillus species. II. The location of genes concerned with the synthesis of ribosomal components and soluble RNA. *Proc Nat Acad Sci USA* 54: 724-730, 1965.
- (32) JONES, K. W.: The role of the nucleolus in the formation of ribosomes. *J Ultrastruct Res* 13: 257-262, 1965.

- (33) WALLACE, H.: Cytological and biochemical studies of anucleolate *Xenopus* larvae. *Quart J Micr Sci* 103: 25-35, 1962.
- (34) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (35) WALLACE, H., and BIRNSTIEL, M. L.: Ribosomal cistrons and the nucleolus organizer. *Biochim Biophys Acta* 114: 296-310, 1966.
- (36) GATES, R. R.: Nucleoli and related nuclear structures. *Bot Rev* 8: 337-409, 1942.
- (37) SIRLIN, J. L.: The nucleolus. *Progr Biophys* 12: 25-66, 1962.
- (38) BEERMANN, W.: Der Nukleolus als Lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.
- (39) MCCLINTOCK, B.: The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
- (40) HEITZ, E.: Nukleolen und Chromosomen in der Gattung *Vicia*. *Planta Med* 15: 495-505, 1931.
- (41) DAS, N. K.: Chromosomal and nucleolar RNA synthesis in root tips during mitosis. *Science* 140: 1231-1233, 1963.
- (42) GUTTES, E., GUTTES, S., and RUSCH, H. P.: Morphological observations on growth and differentiation of *Physarum polycephalum* grown in pure culture. *Develop Biol* 3: 588-614, 1961.
- (43) STEVENS, B. J.: The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J Cell Biol* 24: 349-368, 1965.
- (44) FAVARD, P.: Evolution des ultrastructures cellulaires au cours de la spermatogenese de l'*Ascaris*. *Ann Soc Roy Sci Med Natur Brux* 3: 55-140, 1961.
- (45) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. I. The nucleus of *Bradysia mycorum* Frey (Sciaridae), with special reference to the nucleolus. *J Cell Biol* 17: 153-165, 1963.
- (46) LAFONTAINE, J. G.: A light and electron microscope study of small, spherical nuclear bodies in meristematic cells of *Allium cepa*, *Vicia faba* and *Raphanus sativus*. *J Cell Biol* 26: 1-17, 1965.
- (47) LAFONTAINE, J. G., and CHOUINARD, L. A.: A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J Cell Biol* 17: 167-201, 1963.
- (48) LONGWELL, A. C., and SVIHLA, G.: Specific chromosomal control of the nucleolus and of the cytoplasm in wheat. *Exp Cell Res* 20: 294-312, 1960.
- (49) MILLER, O. L.: Studies on the ultrastructure and metabolism of nucleoli in amphibian oocytes. *In* Fifth International Congress for Electron Microscopy (Breese, S. S., ed.). New York and London, Academic Press Inc., 1962, vol 2, pp NNS.
- (50) PELLING, C.: Ribonukleinsäure-Synthese der Riesenchromosomen. Autoradiographische Untersuchungen an *Chironomus tentans*. *Chromosoma* 15: 71-122, 1964.
- (51) SIRLIN, J. L., and JACOB, J.: Sequential and reversible inhibition of synthesis of ribonucleic acid in the nucleolus and chromosomes: effect of benzamide and substituted benzimidazoles on Dipteran salivary glands. *Nature (London)* 204: 545-547, 1964.
- (52) JACOB, J., and SIRLIN, J. L.: Electron microscope autoradiography of the nucleolus in insect salivary gland cells. *Nature (London)* 202: 622-623, 1964.

PLATE 33

FIGURE 1.—A multinucleate alga, *Vaucheria*, fixed in lead acetate and showing the precipitate localized in the nuclei. Staining reagent H_2S .

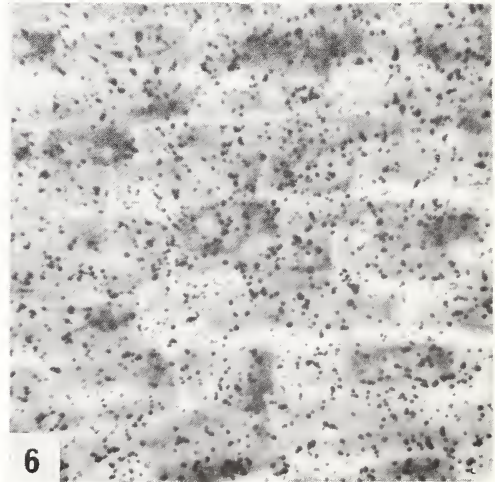
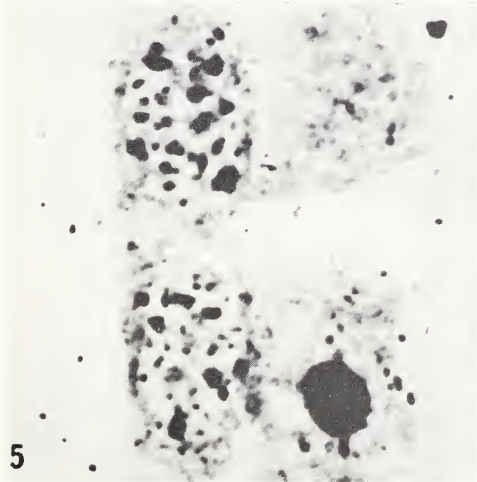
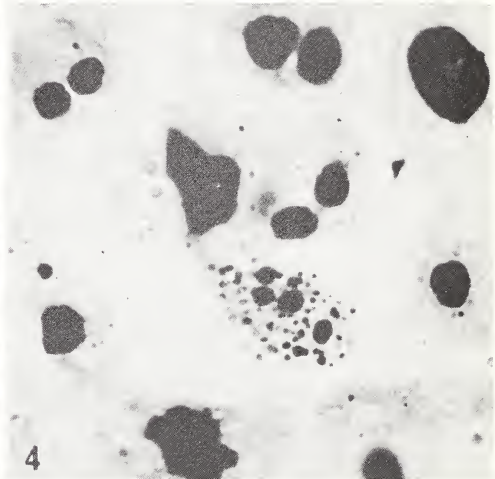
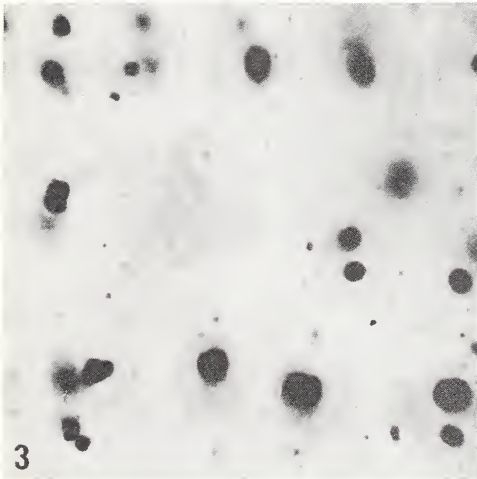
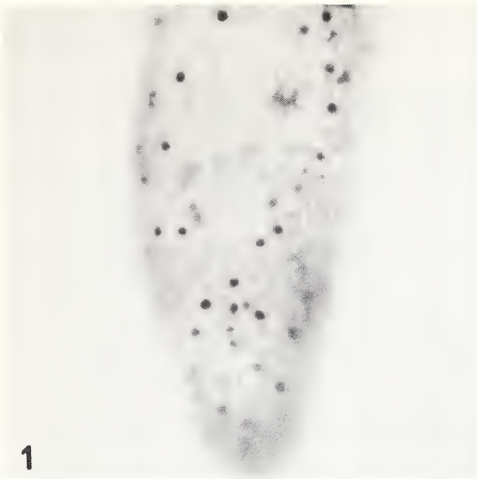
FIGURE 2.—Same as figure 1 but prefixed in ethanol. Absence of precipitates in nuclei.

FIGURE 3.—A section through onion root tip fixed in lead acetate showing the nucleolar localization of orthophosphate. In the center, a cell in metaphase shows lack of precipitate on the chromosomes.

FIGURE 4.—Hot trichloroacetic acid squash of Carnoy-fixed broad bean root tip. Silver staining specifically located to the nucleoli. These nucleoli are lead acetate negative. One telophase nucleus is seen filled with small darkly stained bodies along the chromosomes. *Note* some larger prenucleolar bodies.

FIGURE 5.—Autoradiograph of onion root tip section incubated 1 hour with H^3 -cytidine and stained with silver for nucleoli. Many grains of the emulsion are obscured in this figure by the strong nucleolar staining. Two daughter nuclei are filled with a large number of prenucleolar bodies. They are smaller than the mature nucleolus, but larger than the silver grains of the emulsion layer.

FIGURE 6.—Autoradiograph of onion root tip grown in P^{32} for 2 days and briefly exposed. *Note* absence of grains over many nucleoli.



DISCUSSION

Feinendegen: I think your data may help to explain the differences in opinion regarding the structures found in nucleoli of otherwise comparable cells. I think this also because my own data (*cf* Discussion after Kasten's paper, this Monograph) give evidence that RNA is synthesized in the nucleus and nucleolus at specific chromatin sites that are genetically variable. I do not know yet whether the location of these specific sites depends solely on the stage of the cell within the cell cycle. I believe it is very important to consider the data presented in this session, as an expression or function of a certain stage of nucleolar RNA synthesis during various phases of the cell cycle. If one could look at the nucleolar structure and function during various stages in the cell cycle, some of the discrepancies could be reconciled.

Kasten: With regard to Dr. Feinendegen's question about the timing of RNA synthesis during the cell cycle, we have obtained some autoradiographic data on this point which I will present later. I mention briefly that in our synchronized cell cultures there appears to be continuous uridine incorporation into RNA of nucleoli throughout interphase except for one short time during the S phase. In addition, there is the well-known depression during mitosis. The sudden change during S phase has not been reported before. At the time when incorporation stops there is a concomitant burst in nucleolar DNA synthesis. This occurs at about 3 to 4 hours into the S phase, which would be approaching the mid-S phase.

Love: What is the evidence that the precipitation of lead is due to the presence of orthophosphate? Dr. Studzinski and I (Studzinski and Love, *Stain Techn* 39: 397-401, 1964) have found the same thing. But we also found that no matter how we fixed the cells, lead was no longer specifically precipitated. We were never able to find the reason for this precipitation.

Tandler: The lead-precipitating material is an acid-soluble component, and in root tips more than half of it is orthophosphate. If one makes a chromatogram and stains for lead-precipitating substances, only orthophosphate is found.

Mandel: It should be pointed out that there are phosphorylated compounds, specifically the phosphatidopeptides quickly labeled by P^{32} , which are not extracted by successive treatments with acid and chloroform-methanol. These compounds contain phosphopeptides which are only released by acidic chloroform-methanol (*Biochem Biophys Acta* 90: 408, 1964).

Sirlin: With respect to the P^{32} incorporation into RNA, we have checked on this chemically. We repeated with Dr. Loening exactly the same treatment of the rootlets after P^{32} that we did on the sections with Dr. Tandler (21). After very short periods of label, 15 seconds plus 2 hours chase, 60% of the label is in RNA; label in polyphosphates, phosphoproteins, phospholipids, etc., never rises anywhere near to the RNA level. Dr. Tandler's autoradiographic results showing an enormous amount of radioactive RNA in the cytoplasm compared to the nucleolus are validated chemically.

NUCLEOLAR COMPOSITION

The establishment of a firm chemical basis for the nucleolus has been dependent upon the development of modern biochemical and biophysical techniques. The quantitation of nucleolar components required first the development of procedures for isolation of nucleoli in significant quantity, and then the application of the tools of modern analytical biology. As the study of the nucleolus has only just entered this era, the following papers give numbers to only the most obvious of biological molecules, in particular: RNA, DNA, basic and nonbasic proteins, and certain enzymes. Even the results of the analytical procedures do not agree, as is reported in the two papers on transfer RNA, one of which demonstrates nucleolar synthesis, the other, the lack of nucleolar synthesis of this material. Dr. J. E. Edström was unable to present personally his paper summarizing the ultramicroanalysis of nucleolar composition. Therefore, no discussion follows. This session was chaired by Professors G. Schreiber and P. P. Cohen.

Composition of Nucleoli Isolated From Mammalian Cells^{1, 2}

HARRIS BUSCH, RAOUL DESJARDINS, DONALD GROGAN, KEN HIGASHI, SAMSON T. JACOB, MASAMI MURAMATSU, TAE SUK RO, and WILLIAM J. STEELE,³ *Department of Pharmacology, Baylor University College of Medicine, Houston, Texas*

SUMMARY

The nucleolus is a site of extensive RNA synthesis in nuclei of neoplastic cells, growing and dividing cells, and, to a lesser extent, in other cells. RNA of isolated nucleoli contains 45S, 35S, 28S, and a smaller amount of 6S RNA. Isolated nucleoli contain little 18S RNA by comparison with extranucleolar nuclear RNA which contains mainly 18S, 28S, and 6S RNA. With the aid of actinomycin D, evidence has been obtained that the 45S RNA is converted into 35S and 28S RNA in the nucleolus and that the conversion can be blocked by actinomycin D. Moreover, studies with thioacetamide have shown that 45S and 35S RNA

accumulate largely in nucleoli of thioacetamide-treated animals, and also the amounts of these are considerably larger in animals with regenerating liver. Studies on the turnover time or half-life of 45S RNA with the aid of actinomycin D have indicated that the half-life of this RNA is approximately 8 minutes. By large-scale isolation procedures, base compositions of 45S extranucleolar nuclear and nucleolar RNA have been determined. The nucleolar 45S RNA is GC-rich, *i.e.*, $A + U/G + C = 0.60$, and the extranucleolar is AU-rich, *i.e.*, $A + U/G + C = 1.10$.—*Nat Cancer Inst Monogr* 23: 193–212, 1966.

FOLLOWING THE DEVELOPMENT of procedures for isolation of nucleoli from a variety of plant and other life forms, methods were developed for isolation of nucleoli from mammalian cells (1–4). Initially, these methods were capable of providing only very small quantities of nucleoli so that all analyses were made on micro amounts and further characterization of the components was quite difficult.

In view of the importance of the nucleoli to the problem of neoplastic cells and the functional activity of preneoplastic cells such as those treated with thioacetamide, attempts were made in this laboratory to develop

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This work was supported by grant 8182 from the U.S. Public Health Service and by grants from The American Cancer Society, Inc., The Jane Coffin Childs Fund, The National Science Foundation, and The Anna Fuller Fund.

³ We are grateful to Mrs. Helen Adams, Joe P. Arendell, Charles W. Taylor, and Leroy Hodnett for their excellent technical assistance.

techniques for isolation of nucleoli on a large scale (5, 6). In essence, large-scale mincers were employed as well as continuous homogenizing systems for batch and continuous centrifugation of the products. With the aid of these techniques, sufficient quantities of nucleoli became available so that studies could be made on the content of the nucleic acids, histones, acidic proteins, and the enzymes of the nucleoli (2, 5, 7-9).

The important role of the nucleoli in nuclear metabolism necessitated studies on the composition of nucleolar RNA as well as the other components. It is in the field of the RNA composition and metabolism of nucleoli that there has been the greatest interest thus far. Fortunately the methods of isolation and purification of RNA have permitted a good deal of attention to be devoted to the subject of purification and turnover time of special fractions of nucleolar RNA. Although presently this subject is one of substantial interest, other studies on nucleolar proteins are of importance and may be relevant to the differential functions of nucleoli in neoplastic and other cells.

COMPOSITION OF NUCLEOLI ISOLATED BY THE SONICATION PROCEDURE

Data on the composition of nucleoli are presented in table 1. The isolated nucleoli obtained by the sonication procedure contain substantial amounts of RNA, protein, and DNA. By comparison with nuclei ob-

TABLE 1.—Composition of nucleoli of Walker tumor and liver*

Procedure	DNA (%)	RNA (%)	Protein (%)	RNA/DNA	Total (pg/No.)
Liver nucleoli					
Sonication.....	0.38 (3)	0.38 (3.0)	12.3 (94)	1.0	13.1
Compression-decompression	0.22 (3)	0.55 (7.5)	6.5 (89)	2.5	7.3
Liver nuclei					
Sucrose-calcium.....	12.0 (17)	3.0 (4)	55.0 (79)	0.25	70
Citric acid.....	8.2 (21)	2.3 (6)	28.5 (73)	0.28	39
Walker tumor nucleoli					
Sonication.....	1.7 (7)	1.9 (8)	21.6 (86)	1.1	25.2
Compression-decompression	1.6 (12)	2.2 (16)	9.8 (67)	1.3	13.6
Walker tumor nuclei					
Citric acid.....	13.4 (25)	5.7 (10.4)	39.4 (64)	0.43	53.5

*The values are picograms per nucleolus. The percentages of the total are in parentheses.

tained from normal liver, the nucleoli have a higher RNA/DNA ratio, although substantial amounts of DNA are also present. Both in the normal liver and the Walker tumor nucleoli, the RNA/DNA ratio is approximately 1 and the protein composes approximately 80–90% of the dry weight of the nucleoli. When nucleoli were isolated by the compression-decompression procedure, the RNA/DNA ratio was somewhat higher and the amount of protein was somewhat less.

The various values obtained for elementary compositions of the DNA and histones of the isolated nucleoli are shown in tables 2 and 3. Within the limits of experimental error, there were no significant differences in the values obtained for nuclear and nucleolar DNA or nuclear and nucleolar histones of the Walker tumor and the liver taken as a whole. Although the ramifications of these findings are important with respect to

TABLE 2.—Base composition* of DNA—% total residues

	Adenine	Thymine	Cytosine	Guanine
Tumor				
Nucleus.....	28.3	27.4	20.8	23.2
Nucleolus.....	28.1	27.2	21.3	23.2
Liver				
Nucleus.....	28.3	29.2	21.0	21.5
Nucleolus.....	28.6	28.9	21.4	21.1

*Determined by paper chromatography of the bases, using isopropyl alcohol and HCl (1).

TABLE 3.—Amino acid content of the histones of liver and tumor nucleoli and nuclei (?)

Fraction	Nucleoli*		Nuclei*	
	Liver	Tumor	Liver	Tumor
Alanine.....	11.9	11.0	11.8	10.8
Arginine.....	8.1	8.5	7.4	8.0
Aspartic acid.....	6.0	6.5	6.3	6.9
One-half cystine.....	Trace	Trace	Trace	Trace
Glutamic acid.....	9.7	10.4	9.9	10.5
Glycine.....	9.0	8.9	8.7	8.4
Histidine.....	1.8	1.6	1.6	1.6
Isoleucine.....	4.3	4.1	4.4	4.3
Leucine.....	7.9	7.7	8.0	7.9
Lysine.....	13.5	13.7	13.3	13.7
Methionine.....	0.9	0.5	1.0	0.6
Phenylalanine.....	2.3	2.3	2.4	2.3
Proline.....	5.1	5.3	5.4	5.4
Serine.....	6.1	6.0	5.9	5.9
Threonine.....	4.8	5.4	5.6	5.6
Tyrosine.....	2.1	1.8	1.8	1.6
Valine.....	6.5	6.8	6.3	6.3
Acidic/basic.....	0.7	0.7	0.7	0.7

*The values are percentages of total moles of amino acid recovered and are averages of 3–5 experiments. The average standard deviation from the mean was 5.6%.

the function of histones and of DNA, at present they do not provide a basis for understanding of the nucleolar specialization indicated by the following studies on nucleolar RNA.

COMPOSITION OF NUCLEAR AND NUCLEOLAR RNA

The initial studies on isolated nucleoli revealed that the base composition as determined by optical density was very GC-rich. The values for nuclear aqueous (a) and interphase (i) RNA [isolated according to (10)] are given in table 4, which also shows the composition of the a- and i-RNA of whole nuclei, nucleoli, and extranucleolar nuclear RNA of the Walker tumor and the liver. Table 5 presents the analogous determinations on ^{32}P base compositions (10).

TABLE 4.—Base composition (ultraviolet determination)

Tissue	Adenine (A)	Uracil (U)	Guanine (G)	Cytosine (C)	$\frac{A + U}{G + C}$
Nuclear a-RNA					
Walker tumor.....	18.6	19.0	33.6	28.8	0.60
Liver.....	19.2	23.2	32.2	25.2	0.74
Nuclear i-RNA					
Walker tumor.....	23.4	21.6	29.0	26.0	0.82
Liver.....	23.3	28.2	25.5	23.0	1.06
Nucleolar a-RNA					
Walker tumor.....	15.2	20.4	35.6	28.8	0.55
Liver.....	16.4	21.8	34.1	27.7	0.62
Nucleolar i-RNA					
Walker tumor.....	14.8	21.3	36.2	27.7	0.57
Liver.....	14.6	20.0	34.9	30.5	0.53
S-1 a-RNA					
Walker tumor.....	20.3	19.0	32.8	27.9	0.65
Liver.....	20.6	23.8	30.7	24.9	0.80
S-1 i-RNA					
Walker tumor.....	24.9	25.6	27.5	22.0	1.02
Liver.....	23.6	29.3	27.2	19.9	1.12

TABLE 5.—Base composition (^{32}P)

Tissue	Adenylic acid (A)	Uridylic acid (U)	Guanylic acid (G)	Cytidylic acid (C)	$\frac{A + U}{G + C}$
Nuclear a-RNA					
Walker tumor.....	18.1	20.9	32.9	28.2	0.64
Liver.....	24.6	23.0	29.8	22.6	0.91
Nuclear i-RNA					
Walker tumor.....	19.1	24.3	29.9	26.8	0.76
Liver.....	25.6	24.3	27.1	23.1	0.99
Nucleolar a-RNA					
Walker tumor.....	13.0	21.1	35.5	30.4	0.52
Liver.....	20.5	17.0	38.2	24.3	0.60
Nucleolar i-RNA					
Walker tumor.....	12.9	21.1	33.8	32.3	0.51
Liver.....	21.5	17.5	36.4	24.7	0.64
S-1 a-RNA					
Walker tumor.....	21.9	28.6	26.5	23.0	1.02
Liver.....	26.2	25.1	25.3	23.4	1.05
S-1 i-RNA					
Walker tumor.....	23.6	28.3	24.4	23.7	1.08
Liver.....	30.6	21.6	25.8	22.0	1.09

Although the base compositions of the nucleoli of the Walker tumor and the liver were quite similar when analyzed by optical density, the ^{32}P base compositions differed remarkably in the high content of adenylic acid and in the newly synthesized RNA of liver nucleoli by comparison with the low content of adenylic acid in these nucleolar fractions of the Walker tumor. As shown in tables 6 and 7, this difference from the Walker tumor exists not only with respect to the nucleoli of normal liver but also with the nucleoli of regenerating liver. It was initially believed that the difference between the ^{32}P -labeled RNA of the Walker tumor and the liver may have reflected the high growth rate of the Walker tumor. However, it would now appear that the difference was one of tissue type rather than rate of growth.

TABLE 6.—Base composition of RNA of liver nuclei and nucleoli (ultraviolet determination)*

RNA	Time after partial hepatectomy (hrs)	Adenine	Uracil	Guanine	Cytosine
Nuclear aqueous.....	0	19.2	23.2	32.2	25.2
	6	18.8	23.1	31.8	26.1
	18	17.4	21.5	33.3	27.7
Nuclear interphase.....	0	23.3	28.2	25.5	23.0
	6	22.5	26.5	27.7	23.2
	18	21.2	26.6	27.3	24.7
Nucleolar aqueous.....	0	16.4	21.8	34.1	27.7
	6	16.0	20.7	34.5	28.7
	18	15.6	20.4	35.3	28.5
Nucleolar interphase.....	0	14.6	20.0	34.9	30.5
	6	14.5	21.3	34.7	29.5
	18	14.5	21.3	35.7	30.3
Fraction S-1 aqueous.....	0	20.6	23.8	30.7	24.9
	6	21.4	24.8	28.3	25.3
	18	20.5	23.5	30.0	25.8
Fraction S-1 interphase.....	0	23.6	29.3	27.2	19.9
	6	25.0	27.9	25.3	21.7
	18	23.6	27.4	26.9	22.0

*The values for each purine or pyrimidine are averages of the percentage of total purine and pyrimidine base in the RNA fraction determined by ultraviolet absorption.

TABLE 7.—Base composition (^{32}P) of RNA of liver nuclei and nucleoli*

RNA	Time after partial hepatectomy (hrs)	Adenine	Uracil	Guanine	Cytosine
Nuclear aqueous.....	0	24.6	23.0	29.8	22.6
	6	23.5	20.8	30.4	25.2
	18	19.6	17.4	37.1	25.3
Nuclear interphase.....	0	25.6	24.3	27.1	23.1
	6	28.6	21.8	25.5	23.9
	18	25.2	21.4	28.8	24.4
Nucleolar aqueous.....	0	20.5	17.0	38.2	24.3
	6	20.6	15.4	37.8	26.1
	18	20.0	16.6	38.0	25.2
Nucleolar interphase.....	0	21.5	17.5	36.4	24.7
	6	20.7	16.8	36.0	26.3
	18	19.4	17.0	37.5	26.0
Fraction S-1 aqueous.....	0	26.2	25.1	25.3	23.4
	6	23.1	22.4	33.7	20.6
	18	24.4	23.6	29.9	22.1
Fraction S-1 interphase.....	0	30.6	21.6	25.8	22.0
	6	30.2	22.2	23.6	23.9
	18	28.8	21.9	26.2	23.0

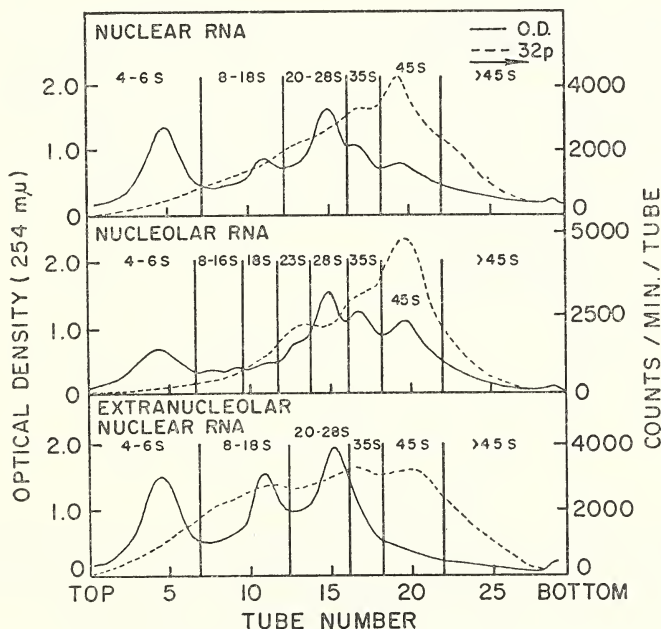
* The values are averages of the percentage of total radioactivity in the RNA fraction that was present in the individual 2',(3')-mononucleotides. Each animal received 1 mc of orthophosphate- ^{32}P intravenously 15 minutes before it was killed.

SUCROSE DENSITY GRADIENT SEDIMENTATION ANALYSIS OF NUCLEAR AND NUCLEOLAR RNA

Text-figure 1 shows the sucrose density gradient sedimentation patterns of RNA of the nuclear and nucleolar fractions of rat liver. These patterns indicate that significant differences exist between the nucleoli and the nuclei with respect to the amounts of 18S and 45S RNA in the patterns. By comparison with the whole nuclei, there is considerably more 35 and 45S RNA in the nucleolar fractions and considerably less 18S RNA. These differences become more marked when the extranucleolar nuclear RNA is studied.⁴

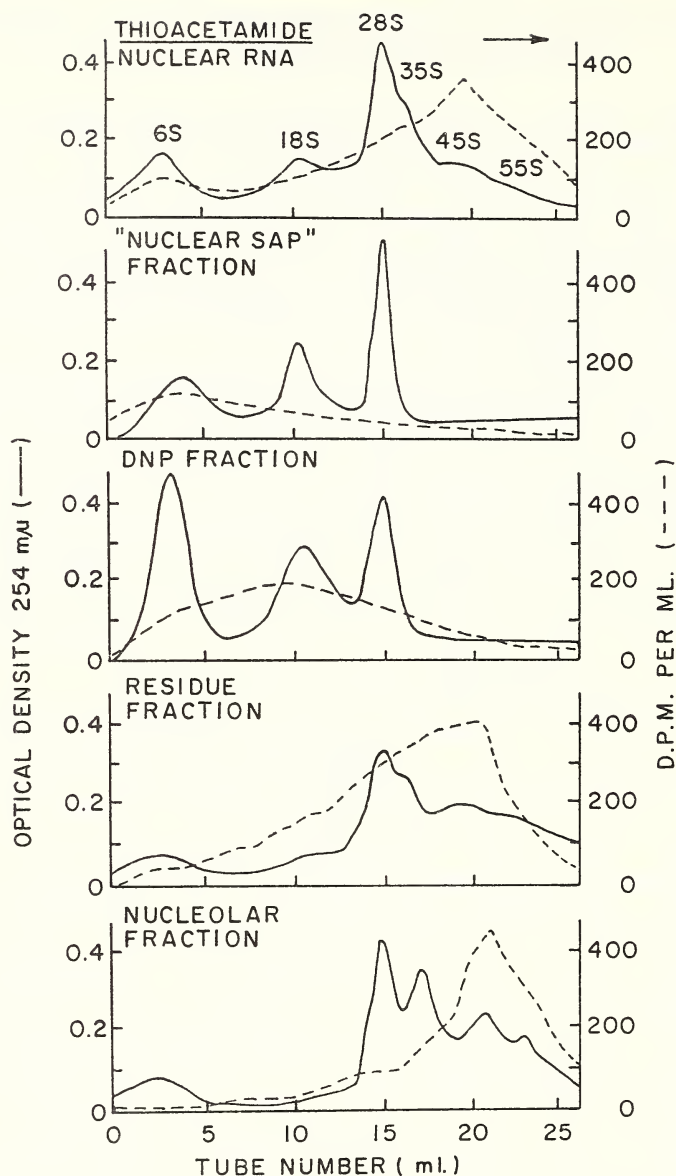
The RNA of the extranucleolar nuclear components is rich in 18S RNA but lacks large amounts of 35, 45, or >45S RNA fractions. As will be shown subsequently, the RNA of this fraction is highly AU-rich by comparison with the nucleolar RNA.

In nucleoli of thioacetamide-treated animals and animals with regenerating livers, the 35 and 45S peaks become much more sharply defined. In addition, in the 55S region of the RNA profile of nucleoli of thioacetamide-treated rats a defined peak may be seen, as shown in text figure 2.



TEXT-FIGURE 1.—Sucrose density gradient sedimentation patterns of RNA of nuclei, nucleoli, and extranucleolar nuclear RNA. ³²P-orthophosphate was injected 30 minutes before the animals were killed.

⁴The sonic-vibrated preparation in 0.25 M sucrose contains nucleoli and broken nuclear components. The sonicate is layered over 0.88 M sucrose and centrifuged at $2000 \times g$ for 20 minutes (11, 12). The most purified fraction is the nucleolar fraction which sediments to the bottom of the tube. The supernatant solutions contain extranucleolar nuclear components.



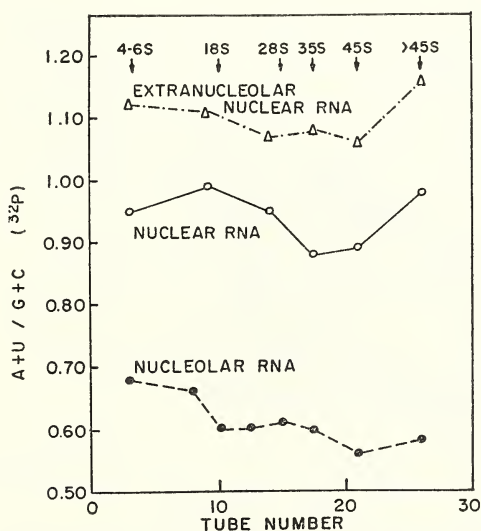
TEXT-FIGURE 2.—Sucrose density gradient sedimentation patterns of RNA of nuclei and nucleolar fractions of animals treated with thioacetamide. ^{32}P -orthophosphate was injected 10 minutes before the animals were killed. Marked enlargements were found in the 55S, 45S, and 35S peaks in the nucleolar fractions.

DISTRIBUTION OF ISOTOPE IN NUCLEOLAR AND EXTRANUCLEOLAR NUCLEAR RNA

As shown in text-figure 1, the RNA initially labeled to the greatest extent is the 45S RNA as has been reported by Darnell, Hiatt, Perry, and others (13-15). The nucleolar RNA contains two peaks of radioactivity and a shoulder at 30 minutes after injection of the isotope. The peak initially labeled is 45S RNA, although RNA of high specific activity is found in all the rapidly sedimenting fractions. A shoulder of 23S RNA is also present, which has a higher degree of labeling. In the extranucleolar RNA, high degrees of labeling are found in the 45S, the 35S, the 8-18S, and the >45S regions. In terms of general specific activity which is probably not very meaningful for this large an aggregation of molecular species, the labeling of the 55S region is probably greatest.

BASE COMPOSITION OF "NEWLY SYNTHESIZED" RNA OF THE NUCLEAR, NUCLEOLAR, AND EXTRANUCLEOLAR NUCLEAR RNA

Text-figure 3 presents the base compositions of fractions of "newly synthesized" nuclear RNA of normal liver with various sedimentation characteristics obtained from studies like those shown in text-figure 1. The nuclear RNA sedimenting in the 35S and 45S regions had a higher GC content than that sedimenting in the >45S and 8-18S regions (text-fig. 3). The "newly synthesized" nucleolar RNA had a high GC content



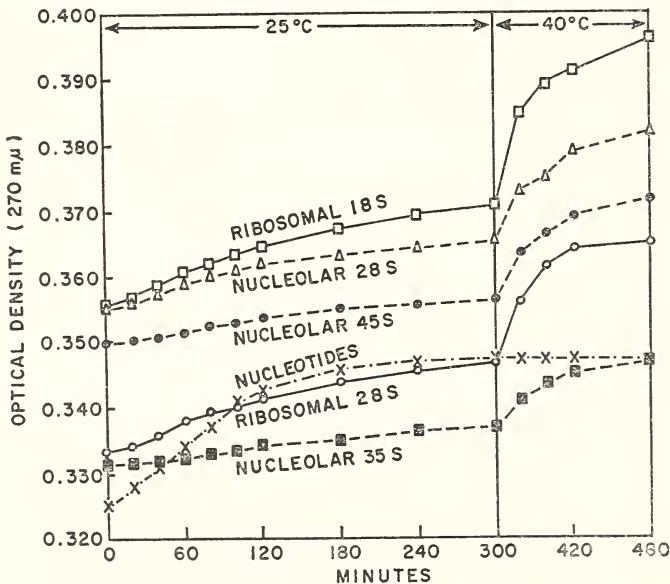
TEXT-FIGURE 3.— ^{32}P base compositions of RNA of various fractions obtained from sucrose density gradients of nuclear RNA fractions.

throughout the sedimentation profile; the "newly synthesized" nucleolar RNA with a lower sedimentation constant was more AU-rich than the nucleolar RNA with higher sedimentation constants. The extranucleolar nuclear RNA had a high AU content throughout the profile. The highest AU content was found in the fraction which sedimented at $>45S$. These data indicate that in the liver nuclei there are at least two classes of 45S RNA, one with a very low A + U/G + C ratio and the other with a ratio reaching approximately 1.15.

REACTION OF RNA WITH FORMALDEHYDE

The reaction of each RNA component with formaldehyde (8, 16) was followed by determination of optical density at $270 m\mu$ for 5 hours at 25 C and for 2 hours at 40 C (8). The formaldehyde was added to a final concentration of 0.5%. Text-figure 4 shows typical patterns of hyperchromicity of each RNA. The reactivity of 45S and 35S RNA was less with formaldehyde than the nucleolar 28S and ribosomal 28S or 18S RNA at both 25 and 40 C. At each temperature the percent increase in optical density at $270 m\mu$ was significantly smaller in the 45S and 35S than the 28S and 18S RNA.

The extent of hydrogen bonding in RNA was calculated from the initial rate of reaction of each RNA with formaldehyde. The reaction rate for the first 60 minutes was compared to that of the nucleotide mixture. This mixture contained 2', 3'-mononucleotides of adenine, uracil, guanine, and



TEXT-FIGURE 4.—Hyperchromicity of RNA fractions after incubation with formaldehyde.

cytosine in the proportion of the base composition of nucleolar RNA (A:16.2, U:20.1, G:34.7, C:29.0). The rate of reaction of ribosomal 28S and 18S RNA was 33 and 37%, respectively, of mononucleotide mixture, indicating that 67 and 63% of amino groups were not available for this reaction, presumably due to hydrogen bonding. These values closely agree with Hall and Doty's estimate of 60–65% for the total microsomal RNA from calf liver (16). The initial rate of reaction of 45S and 35S RNA with formaldehyde was 9 and 7%, respectively, of the mononucleotide mixture. This value suggests that about 90% of the total amino groups of these RNAs are hydrogen bonded. For the nucleolar 28S RNA, the corresponding value was 75% for hydrogen bonding.

HALF-LIFE OF RAPIDLY SEDIMENTING NUCLEOLAR RNA

Studies on the turnover time of 45S RNA of the nuclei and nucleoli of the regenerating liver indicate that the half-life of whole nuclear 45S RNA, as determined by optical density or area under the 45S peak, is approximately 8 minutes. On the other hand, in terms of the area under the 45S peak of radioactivity following an experiment with labeled precursors, the half-life of 45S RNA is approximately 4 minutes (table 8). This value is of interest because the half-life of 45S RNA of the nucleoli of regenerating liver is approximately 4.5 minutes, determined on the basis of optical density. As determined on the basis of radioactivity, the half-life of nucleolar RNA is approximately 4 minutes. For the Walker tumor, the half-life of nucleolar 45S RNA was also determined, and the value on the basis of the optical density under 45S peak is approximately 7 minutes. These data indicate that the rates of release of nucleolar RNA of the Walker tumor, a rapidly growing neoplasm, is intermediate between that of the normal liver and the regenerating liver. The high rates of nucleolar RNA synthesis in the regenerating liver are associated with remarkable increments in the 35S RNA as was reported earlier (17). In addition, these high rates of synthesis are reflected in the substantially greater labeling of RNA in the nucleoli of regenerating liver. What is particularly

TABLE 8.—Half-life of nucleolar 45S RNA*

	Ultraviolet optical density	Orotic acid- ¹⁴ C
Normal liver.....	8–10 minutes...	4
Regenerating liver (18 hours).....	4–5.....	
Thioacetamide-treated liver.....	32.....	
Walker tumor.....	7.....	

*The half-lives were determined by the decrease in area of the 45S peak. Planimetric analysis of the peaks and the total area of the sucrose density gradient sedimentation profile was carried out prior to determination of the 45S peak.

interesting is the rate of transit of this RNA from the nucleolus to other nuclear and cytoplasmic components. Apparently, once the liver cell begins to grow, increased synthetic rates are accompanied by increments in transport rates such that there is a very rapid formation of new cellular products.

From these studies it would seem that 45S nucleolar RNA is one of the fastest turning-over components of the nucleus and of the whole cell, and, moreover, that the rates of turnover are dependent on the physiological needs of cells as well as on their growth rates.

NUCLEOLAR PROTEINS AND ENZYMES

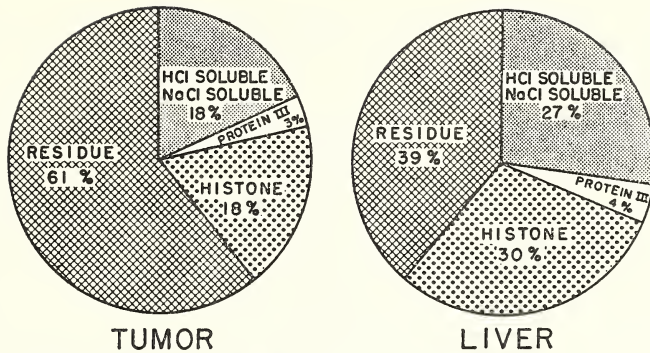
The subject of "nucleolar enzymes" will be dealt with by Dr. Seibert who will report on studies initiated in this laboratory. However, two enzymes particularly involved in RNA metabolism are concentrated in large measure in the nucleolus, namely, RNA polymerase (9, 11) and ribonuclease (9, 18, 19). Although ample data are available on the composition and quantities of nucleolar RNA, little information is available on these enzymes, presumably because they are bound to nucleolar structures. As nucleoli are prepared, nucleolar RNA polymerase and nucleolar ribonuclease are not rendered soluble in the course of isolation of nucleoli but rather remain adherent to nucleolar components until they are released by more drastic procedures.

In addition to these enzymes, nucleoli must contain a relatively specific enzyme that we refer to as a "convertase," which is involved in the orderly transition of rapidly sedimenting RNA of the 45 and 55S regions to 35 and 28S RNA. At present, nothing is known about this enzyme, particularly whether the specificity of the types of products produced is related to the specificity of the enzyme or to the nature of the substrate. The actions of this enzyme will have to be studied because of the uncertainties about its effects on the substrate, namely, whether it is a reductive enzyme, an enzyme that influences hydrogen bonding, or a special cleavage enzyme such as a highly specific ribonuclease.

ACIDIC NUCLEOLAR PROTEINS

It was pointed out in earlier studies, summarized recently (20), that in the aggregate the nucleolar proteins are of the acidic type. As discussed earlier in this study, the nucleolar histones are similar in composition to the nuclear histones. As indicated in text-figure 5, they compose a relatively small percentage of the total nucleolar protein, *i.e.*, approximately 25-30% in liver and only slightly less in the tumor.

Table 9 presents the amino acid composition of the fractions obtainable from nucleolar proteins of the Walker tumor. These fractions are: (a)



TEXT-FIGURE 5.—Percentages of total nucleolar protein accounted for by histones, saline-soluble proteins, and residual proteins. Protein III is the fraction soluble in 0.15 M NaCl and insoluble in HCl.

soluble in 0.15 M sodium chloride, (b) soluble in 0.25 N HCl, and (c) insoluble in either dilute saline or HCl. This residual fraction of the nucleolar proteins represents a substantial part of the protein present in the tumor nucleoli, but somewhat less in the nucleoli of normal liver cells.

The amino acid composition of these fractions (table 9) is not remarkable although arginine content is low and the content of lysine is also relatively low both in the proteins insoluble in 0.25 N HCl and 0.15 M NaCl as well as in the protein soluble in 0.15 M NaCl. The content of cysteine is appreciable, and it is possible that the cysteine content may reflect the

TABLE 9.—Amino acid content of proteins obtained by fractionating liver nucleoli

Fraction	Nucleoli			
	NaCl-soluble	NaCl-HCl-soluble	NaCl-soluble, HCl-insoluble	HCl residue
Alanine.....	9.1	9.2	6.5	7.9
Arginine.....	3.9	3.6	2.9	5.5
Aspartic acid.....	10.2	11.0	11.0	9.1
One-half cysteine.....	1.9	1.9	1.6	1.4
Glutamic acid.....	11.3	11.7	14.0	12.8
Glycine.....	9.0	8.5	8.2	8.6
Histidine.....	2.9	2.3	1.6	2.1
Isoleucine.....	4.3	3.7	4.6	4.4
Leucine.....	9.7	8.5	10.9	9.5
Lysine.....	8.1	8.7	4.7	7.8
Methionine.....	1.0	1.6	1.7	1.4
Phenylalanine.....	4.6	3.3	3.5	3.5
Proline.....	5.7	6.2	5.1	5.8
Serine.....	6.6	6.3	7.6	7.7
Threonine.....	4.6	4.7	4.9	4.4
Tyrosine.....	1.7	1.3	2.0	1.6
Valine.....	7.3	6.4	4.6	6.5
Acidic/basic.....	1.4	1.6	2.7	1.4

* The values are percentages of total moles of amino acid recovered and are averages of 3-5 experiments. The value for glycine was corrected for nucleic acid present. The average standard deviation from the mean was 8.0%.

concentration of sulfhydryl groups reported by Albertini (21). Further studies are in progress on the concentration of sulfhydryl groups in nucleoli in our laboratory.

DISCUSSION

These data, along with others, support the concept that $45S \rightarrow 35S \rightarrow 28S$ reflects the pattern of nucleolar synthesis of RNA. The ultraviolet base compositions of these 3 RNA fractions are similar, and the principal difference may be one of hydrogen bonding.

Differences have been found between the ^{32}P base composition and ultraviolet base composition of the extranucleolar nuclear RNA and the nucleolar RNA. The extranucleolar nuclear RNA fractions are poly-disperse under the conditions of their isolation, but they are all AU-rich by comparison with the nucleolar fractions. Marked differences have been found between Walker tumor and liver with respect to the adenylic acid content of newly synthesized nuclear and nucleolar RNA (10, 22).

The evidence provided in the studies indicates that 28S RNA is a product of the nuclei but 18S RNA is apparently an extranucleolar product. Presumably, the 30S ribosomal component is added to the nucleolar 60S RNP component as it moves through the nuclear ribonucleoprotein network.

RESUMEN

El nucleodo es un sitio de extensa síntesis de ARN en núcleos de células neoplásicas, células en división y en crecimiento, y en menor extensión, de otras células. El ARN de los nucleolos aislados contienen ARN 45S, 35S, 28S y una cantidad menor de ARN 6S. Los nucleolos aislados contienen poco ARN 18S, 28S y 6S. Con la ayuda de la actinomicina D se han obtenido pruebas de que el ARN 45S se convierte, en el nucleolo, en ARN 35S y 28S y que la conversión puede ser bloqueada por la actinomicina D. Además los estudios realizados con tioacetamida han mostrado que los ARN 45S y 35S se acumulan en grandes cantidades en los nucleolos de los animales tratados con tioacetamida, y, también, que las cantidades de éstos son considerablemente mayores en los animales con hígado en regeneración. Los estudios del tiempo de transformación, o sea la vida media del ARN 45S con la ayuda de la actinomicina D han indicado que la vida media de este ARN es de aproximadamente 8 minutos. Mediante procedimientos de aislamiento en gran escala se han determinado las composiciones básicas del ARN 45S extranucleolar nuclear y nuclear. El ARN 45S nucleolar es rico en GC, esto es, $A + U/G + C = 0.60$, y el extranucleolar es rico en AU, esto es, $A + U/G + C = 1.10$.

REFERENCES

- (1) BUSCH, H., MURAMATSU, M., ADAMS, H. R., SMETANA, K., STEELE, W. J., and LIAU, M. C.: Isolation of nucleoli. *Exp Cell Res Suppl* 9: 150-163, 1963.
- (2) MURAMATSU, M., SMETANA, K., and BUSCH, H.: Quantitative aspects of isolation of nucleoli of Walker carcinosarcoma and liver of the rat. *Cancer Res* 23: 510-518, 1963.

- (3) DESJARDINS, R., SMETANA, K., STEELE, W. J., and BUSCH, H.: Isolation of nucleoli of the Walker carcinosarcoma and liver of the rat following nuclear disruption in a French pressure cell. *Cancer Res* 23: 1819-1823, 1963.
- (4) MAGGIO, R., SIEKEVITZ, P., and PALADE, G.: Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic sub-fractions. *J Cell Biol* 18: 293-312, 1963.
- (5) DESJARDINS, R., SMETANA, K., and BUSCH, H.: A large scale procedure for isolation of nucleoli from rat liver. *Exp Cell Res* 40: 127-137, 1965.
- (6) DESJARDINS, R., SMETANA, K., GROGAN, D., HIGASHI, K., and BUSCH, H.: A large-scale procedure for isolation of nucleoli from the Walker tumor. *Cancer Res* 26: 97-104, 1966.
- (7) GROGAN, D., DESJARDINS, R., and BUSCH, H.: Nucleolar proteins of rat liver and Walker tumor. *Cancer Res* 26: 775-779, 1966.
- (8) MURAMATSU, M., HODNETT, L., and BUSCH, H.: Base composition of fractions of nuclear and nucleolar RNA obtained by sedimentation and chromatography. *J Biol Chem* 241: 1544-1550, 1966.
- (9) SIEBERT, G., VILLALOBOS, J., RO, T. S., STEELE, W. J., LINDENMAYER, G., ADAMS, H., and BUSCH, H.: Enzymatic studies on isolated nucleoli of rat liver. *J Biol Chem* 241: 71-78, 1966.
- (10) MURAMATSU, M., and BUSCH, H.: Studies on nucleolar RNA of the Walker 256 carcinosarcoma and the liver of the rat. *Cancer Res* 24: 1028-1034, 1964.
- (11) RO, T. S., and BUSCH, H.: *In vitro* labeling of RNA in isolated nucleoli of the Walker tumor and liver. *Cancer Res* 24: 1630-1633, 1964.
- (12) RO, T. S., MURAMATSU, M., and BUSCH, H.: Labeling of RNA of isolated nucleoli with UTP ^{14}C . *Biochem Biophys Res Commun* 14: 149-155, 1964.
- (13) SCHERRER, K., LATHAM, H., and DARNELL, J. E.: Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 49: 240-248, 1963.
- (14) HIATT, H. H.: A rapidly labeled RNA in rat liver nuclei. *J Molec Biol* 5: 217-229, 1962.
- (15) PERRY, R. P.: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat Cancer Inst Monogr* 14: 73-87, 1964.
- (16) HALL, B. D., and DOTY, P.: The preparation and physical chemical properties of ribonucleic acid from microsomal particles. *J Molec Biol* 1: 111-126, 1959.
- (17) MURAMATSU, M., and BUSCH, H.: Studies on nuclear and nucleolar ribonucleic acid of regenerating rat liver. *J Biol Chem* 240: 3960-3966, 1965.
- (18) VILLALOBOS, J., JR., STEELE, W. J., and BUSCH, H.: Increased ribonuclease activity of nuclei of liver cells of thioacetamide-treated rats. *Biochem Biophys Res Commun* 17: 723-729, 1964.
- (19) VILLALOBOS, J., JR., STEELE, W. J., and BUSCH, H.: Increased ribonuclease activity of isolated nucleoli of livers of thioacetamide-treated rats. *Biochim Biophys Acta* 103: 195-200, 1965.
- (20) BUSCH, H.: Histones and Other Nuclear Proteins. New York, Academic Press Inc., 1965.
- (21) ALBERTINI, DE M.: Etude cytophotométrique des protéines-SH nucléolaires de la racine de Jacinthe; répartition précise de ces composés au sein de nucléole. *CR Acad Sci (Paris)* 248: 3476-3478, 1959.
- (22) OKAMURA, N., and BUSCH, H.: Base composition of high molecular weight nuclear RNA of Walker tumor and liver of the rat. *Cancer Res* 25: 693-697, 1965.

DISCUSSION

Perry: What percentage of the total nuclear DNA is associated with your nucleoli? With respect to your evidence that the 18S RNA is not part of the nucleolar 45S RNA component, do you find the same base composition with short pulses of radioactivity as with ultraviolet absorbance? As Dr. Penman and I will discuss, the 18S RNA component moves out very rapidly, and this could account for the apparent failure to detect an 18S RNA contribution to the 45S RNA base composition.

Busch: Regarding the question as to the percentage of the total nuclear DNA in the nucleolar preparation, our procedure results in precipitation of nucleoli and also the perinucleolar chromatin which Dr. Bernhard demonstrated. The DNA in our preparations is approximately 5% of the whole nuclear DNA.

Figure 3 shows that with a 10- or 20-minute pulse of ^{32}P -orthophosphate, the extranucleolar nuclear RNA of liver has a ratio of $\text{A} + \text{U}/\text{G} + \text{C}$ of about 1.17 with a maximum in the 55S RNA, the whole nuclear RNA has an $\text{A} + \text{U}/\text{G} + \text{C}$ ratio of about 0.95, and the nucleolar RNA has an $\text{A} + \text{U}/\text{G} + \text{C}$ ratio of about 0.6. The accompanying table shows that the 45S, 35S, and 28S nucleolar RNAs have (within the limits of the error of our methods) identical base compositions. Although the nucleolus may make more than one RNA, the predominant product is 28S RNA. Evidence with actinomycin D blocks indicated that there is another component, somewhat richer in AU. In the case of the Walker tumor, an RNA with an even lower $\text{A} + \text{U}/\text{G} + \text{C}$ ratio than that present in liver is found.

Base composition (ultraviolet) of nucleolar RNA of normal rat liver

Species	Adenine	Uracil	Guanine	Cytosine	$\frac{\text{A} + \text{U}}{\text{G} + \text{C}}$
45S.....	14.5	20.8	35.3	29.4	0.55
35S.....	14.3	19.8	35.1	30.8	0.52
28S.....	15.0	19.9	34.9	30.2	0.54
6S.....	20.5	28.6	28.9	22.0	0.96

With the ^{32}P studies, the newly synthesized RNA has a higher adenine and a lower uracil content as compared with the ultraviolet studies in liver; in the Walker tumor the values for both are very similar. In both cases, the over-all base composition, ratio of $\text{A} + \text{U}/\text{G} + \text{C}$, is virtually identical.

Brown: Were all of the curves you showed from *in vivo* labeling with subsequent isolation of nucleoli?

Busch: Yes.

Brown: What are some of the physical and chemical characteristics of the RNA synthesized by the purified nucleolar fraction?

Busch: With the isolated nucleoli we cannot get net RNA synthesis. That is to say, we have tried a variety of systems including addition of ribonuclease inhibitors and various kinds of nucleoli from thioacetamide and other kinds of sources, but we do not get net RNA synthesis.

Brown: What do you mean by net RNA synthesis? Is there isotope incorporation?

Busch: Isotope incorporation is not synthesis. This lax terminology is common among "molecular biologists" and some biochemists. Many workers assume that addition of the radioactive component to a macromolecule constitutes "synthesis," but it may not be so. If one starts out with 50 to 100 μg or pg of RNA, and at the end of the reaction 25 are left but are labeled, some report there is synthesis. As far as we are concerned, this is not synthesis.

Brown: But from your nearest neighbor's studies, it is clear that there is incorporation into some kind of a polynucleotide chain. Presumably your non-net synthesis is due to simultaneous degradation.

Busch: That's right. What is required is an appropriate system to produce net synthesis of RNA such as that recently reported by Spiegelman (Spiegelman, *et al.*, Proc Nat Acad Sci USA 54: 919-927, 1965).

Brown: You previously commented on the fact that you had isolated an enzyme that could cleave the 45S RNA to smaller segments. Would you comment further on this?

Busch: We have studied the nucleolar ribonucleases, but these have different activities from the "convertases" which may not cleave the RNA. We have no enzyme for normal cleavage of 45S RNA: our data suggest there are changes in secondary or tertiary structure of the RNA. Thus far, we do not have a purified "convertase."

von Borstel: Do you have any idea where the 6S RNA in the nucleoli comes from?

Busch: No, but interestingly, 6S RNA is not readily labeled with either ^{32}P -phosphate or orotic acid. This fraction must turn over very slowly.

Swift: I'd like to ask one question about the 3 minutes you allotted to morphology. What is the "perinucleolar network"? Do you consider this as being a specific structure in your cells, or is it merely, as I suspect, the nucleoplasm in between the more condensed regions of chromatin?

Busch: The accompanying electron micrographs (figs. 1 and 2) were obtained by Dr. Smetana and Dr. Shankar Narayan of our laboratory and show that at the border of the nucleolus, or at the junction with the extranucleolar nucleoprotein, apparently some substances combine to form a kind of network. This network, which can be seen both by electron microscopy and light microscopy, is much larger in the Walker tumor than in the liver. To demonstrate this network, the nuclei must be treated with deoxyribonuclease or with 2 M sodium chloride to extract the deoxyribonucleoprotein.

Sirlin: Does it include also the rim around the nucleolus that comes along in your isolation?

Busch: In the isolated nucleoli only the perinucleolar chromatin remains attached. We are trying very hard to learn what the composition of this DNA is and to develop methods for isolation of the RNP network.

Mandel: I'm not sure that 45S RNA is hydrogen bonded as you suggest. We have studied this by gradient sedimentation in urea. The 45S RNA was in 6 to 4 M urea during centrifugation and it still remained 45S. If it had been connected by hydrogen bonds it should have been degraded. [Bull Soc Chim Biol (Paris), 1966, in press].

Bernhard: Dr. Busch has done exactly what all of us have to do now, namely, to try to reconcile biochemical data with morphological findings. But I personally feel that it is a little dangerous to move too rapidly. We do not yet know if the 28S RNA really is localized in the particulate component of the nucleolus. These are not RNA granules, these are RNP granules which have a complex but not well-defined structure. Secondly, I do not think that after actinomycin D treatment the fibrillar area increases. It only seems to increase because fibrils from the whole nucleolar body are sorting. Most of these fibrils are normally masked. In addition, the granular portion is frequently dispersed into the nucleoplasm whereas the fibrillar part is left.

Busch: That the "granules" contain 28S RNA is only a suggestion, for we have not yet isolated the nucleolar granules. With regard to the fibrillar elements, I think the evidence is really pretty good that it increases relative to the 45, 35, and 28S RNA in the actinomycin D-treated liver nucleoli. There is a relative loss of granular elements, as compared to fibrillar elements. Our data from planimetric analysis establishes this quite well in a very large series of observations.

Penman: I look forward to the opportunity of discussing with you the apparent discrepancy of your results with three independent experiments that I shall describe which suggest the 45S is a precursor to 35S plus 18S RNA. I gather that you don't feel this occurs.

Busch: In the nucleolus, 45S RNA is a precursor of 35S RNA which is then a precursor of 28S RNA. The possibility that the 18S RNA is formed from the extra-nucleolar 45S RNA is not ruled out. The extranucleolar 45S RNA is very polydisperse. From our most recent data (Steele and Busch, *Biochim Biophys Acta* 119: 501-509, 1966) it would appear that the 18S RNA may be formed in the nuclear RNP network.

Birnstiel: Dr. Busch's finding of 28S RNA in the nucleolus and Dr. Bernhard's suggestion that the RNA is present in the form of RNP particles are both compatible with results we obtained previously (Birnstiel, Chipchase, and Hyde, *Biochim Biophys Acta* 76: 454-462, 1963). We isolated from pea nucleoli a heterogeneous population of RNP particles containing a surprisingly high proportion of 60S particles which in the cytoplasm correspond to aggregates of 28S RNA and ribosomal protein.

Barr: Do you have any further data on nucleolar proteins?

Busch: We have been very interested in the nucleolar proteins. Our studies on nucleoli began with the false impression that the nucleoli were the site of synthesis of histones, but this does not appear to be so. The nucleolar histones of the Walker tumor and the liver are apparently identical in composition to the whole nuclear histones (*see* table 3) as there are no significant differences in the starch gel electrophoretic patterns. The nucleolar proteins are separated into three major groups: (a) soluble in 0.15 M sodium chloride (table 9), (b) soluble in 0.25 N HCl (containing the nucleolar histones of table 3), and (c) the residual fraction insoluble in either dilute saline or HCl (table 9). The group in (a) is separable into two fractions (table 9): (i) those soluble in 0.15 M NaCl and 0.25 N HCl and (ii) those soluble in 0.15 M NaCl and insoluble in 0.25 N HCl. The residual fraction of the nucleolar proteins represents a substantial part of the protein present in the tumor nucleoli, *i.e.*, about 60%, but only about 40% in the nucleoli of normal liver cells. The amino acid composition is on the acidic side, some 22% of the residues are glutamic and aspartic acid; very small amounts are present of arginine, 4%, and lysine, 8.5%. These proteins are referred to as acidic proteins, since the ratio of acidic to basic amino acids is 1.8. The proteins soluble in 0.15 M NaCl are more readily studied because of their solubility. Some have mobilities on starch gel electrophoretograms similar to those of the histones (Grogan *et al.*, *Cancer Res*, 1966).

Feinendegen: In your autoradiograms using ^3H -actinomycin D, did you always observe the ring pattern of the label around the nucleolus or were there other labeling patterns also? Did you select the ring pattern for demonstration?

Busch: In the Walker tumor under these conditions, there was not a very good perinucleolar or intranucleolar localization of labeled actinomycin D; but in the liver, which is susceptible to actinomycin D, there was a beautiful perinucleolar localization. About 30% of the grains were distributed in this way. There were also more diffuse patterns, of course.

Pelling: My question concerns the DNA you found in the nucleolus. The nucleolar-organizing region proper ought to comprise much less than 5% of the total DNA. In your case, the most probable explanation would be that you are dealing not only with the DNA of the nucleolus organizer, but also with some additional chromatin, presumably from chromosome segments close to the nucleolus. Have you compared the base composition of this "nucleolar" DNA with the total nuclear DNA and the nucleolar RNA?

Busch: The base composition of the total nucleolar DNA is identical to the base composition of the total nuclear DNA. We do not find any significant difference. However, base composition alone tells relatively little.

PLATES

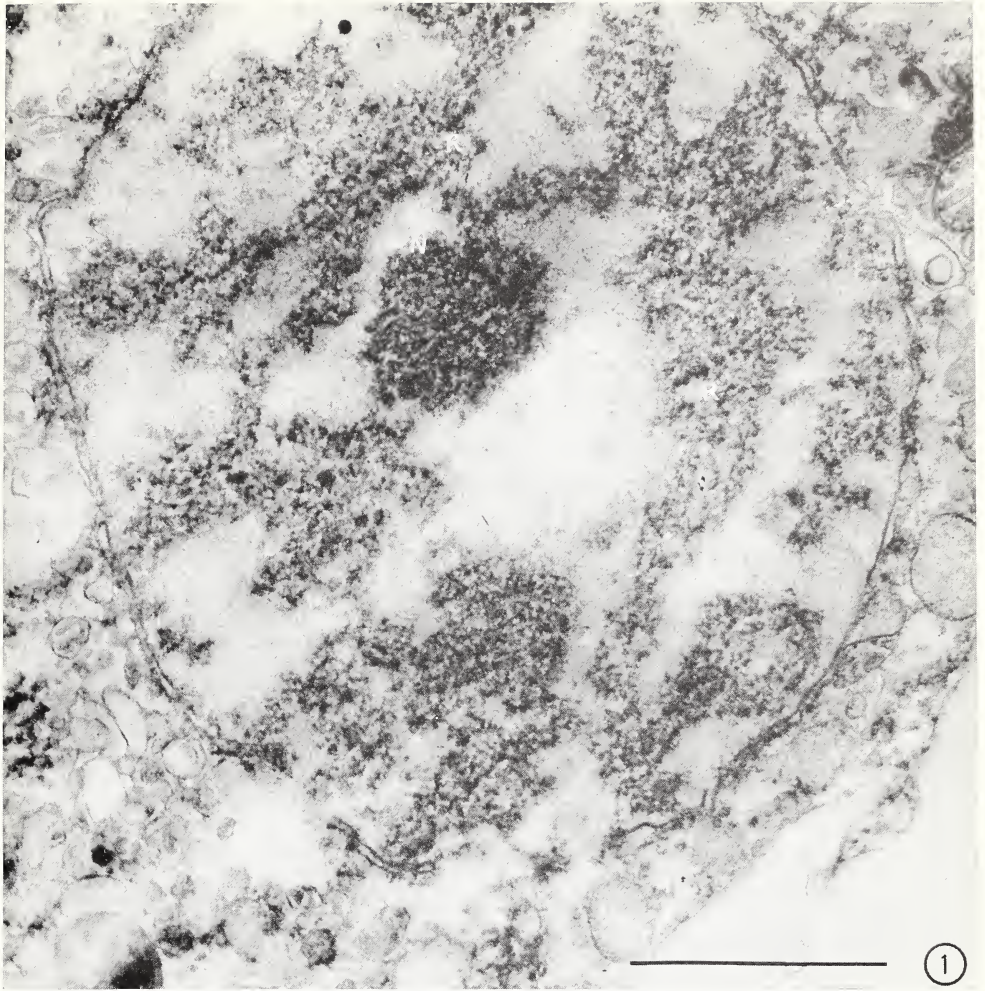


FIGURE 1.—Nucleus of a Walker tumor cell after extraction of deoxyribonucleoproteins by treatment with 2 M NaCl. N: nucleolus; R: ribonucleoprotein network.
× 49,000

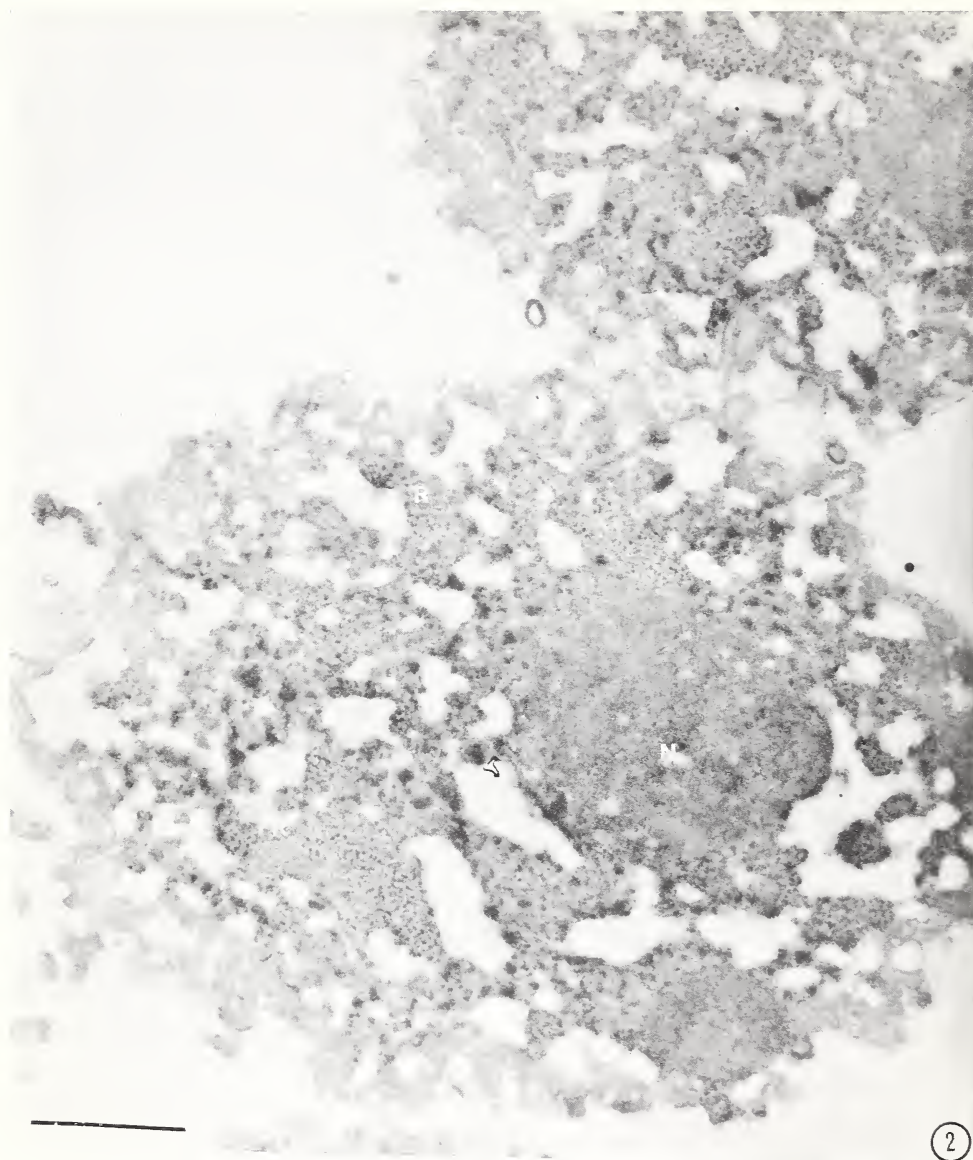


FIGURE 2.—Isolated liver cell nucleus after incubation with deoxyribonuclease. Dense granules of the ribonucleoprotein network (R) appear embedded in amorphous material. N: nucleolus. $\times 31,000$

Progress Report on the Characterization of Nucleoli From Guinea Pig Liver¹

RACHELE MAGGIO,² *Laboratory of Comparative Anatomy, The University, and Research Unit for Molecular Embryology of the Consiglio Nazionale delle Ricerche, Palermo, Italy*

SUMMARY

Nuclei and nucleoli of guinea pig liver have been studied for their ability to carry out incorporation of labeled amino acids into proteins in an *in vitro* system. The results indicate that, whereas in the nuclei incorporation depends on the addition of cofactors, nucleoli not only do not require these cofactors but are inhibited by them. The incorporating activity of isolated nucleoli is much higher than that of the nuclei. Incorporated label was found distributed in both *basic* and *nonbasic* nucleolar protein fractions,

the former showing a higher specific activity. Basic proteins, extracted from nuclei and subnuclear fractions (nucleoplasm I and II and nucleoli), were analyzed by disc electrophoresis to ascertain if there were any damage to proteins as a result of the isolation procedure. In the electrophoresis of nuclear subfractions, complete disappearance of the fast-moving bands suggested some damage which was probably caused by sonication.—*Nat Cancer Inst Monogr* 23: 213–222, 1966.

SUCCESS IN isolation of nucleoli has contributed to our knowledge of the chemical composition and functions of this structure. However, isolation of nucleolus requires drastic procedures, *e.g.*, disruption of the nucleus by sonic vibrations. Hence, when such methods are used it becomes imperative to ascertain whether and to what extent the nucleolus has been damaged with respect to its biochemical activities. The present communication describes experiments aimed at answering this question. As an estimation of biological activity of isolated nucleoli, their ability to incorporate amino acids *in vitro* was measured.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² I wish to express my deep gratitude to Dr. A. Monroy for his valuable suggestions and discussions, Dr. H. Lehrer for reading the manuscript, Dr. G. Millonig for his valuable assistance with the electron microscopy, and Mr. A. O. Oliva for technical help.

ISOLATION OF NUCLEI AND NUCLEAR SUBFRACTIONS

The method for isolation of nuclei from guinea pig liver has been described in detail by Maggio, Siekevitz, and Palade (1). It involves homogenization of the tissue in 0.88 M sucrose containing 1.5 mM CaCl_2 , and centrifugation in a discontinuous density gradient. Electron microscopical observation shows that such nuclear preparations are characterized by negligible cytoplasmic contamination, while the nuclear envelope, chromatin masses, and nucleoli retain the characteristics they have *in situ*. The nuclear fraction was submitted to sonic treatment essentially according to the technique already described (2) and from the sonicated suspension three nuclear subfractions were obtained by centrifugation in a discontinuous sucrose density gradient: a nucleolar subfraction consisting mainly of nucleoli surrounded by a variable amount of nucleolus-associated chromatin and contaminated by chromatin blocks derived primarily from the nuclei of the von Kupffer's cells; two nucleoplasmic subfractions (I and II) consisting of chromatin in a coarser (I) or finer (II) degree of fragmentation. The chemical composition of these nuclear subfractions has been described and their RNA's, in terms of nucleotide composition and *in vivo* turnover, have been characterized (2).

ABILITY OF ISOLATED NUCLEI AND NUCLEOLI TO CARRY OUT INCORPORATION OF LABELED AMINO ACID INTO PROTEINS

Nuclei isolated from guinea pig liver can incorporate, *in vitro*, amino acids into proteins, provided they are supplemented with adenosine triphosphate (ATP), an ATP regenerating system, and cell sap. In the sonicated nuclei this incorporating activity is completely lost (table 1). Pretreatment of nuclei with deoxyribonuclease or ribonuclease inhibits their endogenous activity by about 50%. The inhibitory effect of puromycin is rather small, as was also observed in the pea nucleoli (3). However, whether this result is due to the inability of puromycin to penetrate the nucleus or whether this represents the specific inhibition of only one part of the total nuclear protein synthesis is not known.

Isolated nucleoli can incorporate, *in vitro*, amino acids in the absence of any added cofactors (table 2). Furthermore, the specific activities obtained under these conditions are about 10 times higher than those observed with nuclei plus cofactors (*see* table 1). Kinetic experiments show that the incorporation proceeds very rapidly and a plateau is reached in about 5 minutes, at 37 C. Between 5 and 10 minutes of incubation incorporated counts decrease slightly; the significance of this finding is obscure. The incorporated label is found both in the 0.1 N HCl extract and in the nucleolar residual proteins; the latter fraction shows lower specific

TABLE 1.—Characterization of C¹⁴ amino acid incorporation into isolated nuclei

Experi- ment No.		Condition of experiment	Specific activity of protein (cpm/mg)
1	Intact nuclei.....	Complete system (L-leucine C ¹⁴)..... minus ATP, ATP regenerating system.	152 60
		minus ATP, ATP regenerating and cell sap.	16
	Sonicated nuclei.....	Complete system (L-leucine C ¹⁴)..... minus ATP, ATP regenerating system.	0 0
2	Intact nuclei.....	Complete system (C ¹⁴ AA mix).....	316
		preincubated with 100 μ g deoxy- ribonuclease.	165
		preincubated with 100 μ g ribonu- clease.	141
		preincubated with 100 μ g puro- mycin.	250

The incubation mixture contained in 1 ml: 25 μ moles Tris buffer, pH 7.6; 170 μ moles sucrose; 4 μ moles NaCl; 4 μ moles ATP; 10 μ moles PEP; 10 μ g PEP-kinase and about 2 mg of protein from the guinea pig liver 105,000 \times *g* supernatant (cell sap). Radioactive amino acids used were: L-leucine-C¹⁴, 1 μ c (150 μ c/ μ moles) or a mixture which contained a total of 1 μ c of the following C¹⁴ amino acids: L-phenylalanine (9.1 μ c/ μ moles), lysine (43.8 μ c/ μ moles), L-valine (26 μ c/ μ moles), L-glutamic acid (8.2 μ c/ μ moles), L-leucine (10.7 μ c/ μ moles), and L-alanine (15.2 μ c/ μ moles). In experiment 1, 0.9 mg and in experiment 2, 0.4 mg of nuclear protein were used. Where sonicated nuclei were used, sonication was carried out for 10 minutes at about 10,000 cycles per second. In experiment 2, the nuclei were preincubated with ribonuclease, deoxyribonuclease, or puromycin for 10 minutes at 37 C in 0.88 M sucrose. The samples were incubated in the presence of C¹⁴ amino acids for 30 minutes at 37 C and the reaction terminated by the addition of a solution containing 60 μ moles of C¹² amino acids and 0.4 ml of 50% trichloroacetic acid (TCA). The precipitate, after being washed with cold TCA, was extracted with hot (90 C) 5% TCA and then with a mixture of alcohol-ether (3 : 1). It was then dissolved in ammonia, dried in the vials, and the radioactivity determined with a Nuclear Chicago Scintillation Spectrophotometer.

activity. The addition of cofactors, such as ATP and cell sap, reduces the incorporation of amino acids into the nucleolar residual proteins while completely inhibiting the incorporation into the HCl extractable proteins.

Birnstiel and Hyde (3) found that isolated pea nucleoli rapidly incorporate *in vitro* leucine-C¹⁴ into acidic and basic compounds; such incorporation is stimulated by the addition of an energy-regenerating system and is not dependent on nuclear pH 5 enzymes. They found that the bulk of the incorporated label is recovered in the residual nucleolar proteins, while a small fraction is found in the acid-extractable basic proteins.

TABLE 2.—*In vitro* incorporation of C¹⁴ amino acids into nucleolar protein fractions

Incubation system	Fraction	Specific activity of protein (cpm/100 μ g)
Nucleoli alone.....	Whole nucleoli	260
	0.1 N HCl extract	244
	Residue.....	157
Nucleoli complete.....	Whole nucleoli	76
	0.1 N HCl extract	0
	Residue.....	45

The incubation mixture contained in 1 ml: 10 μ moles Tris buffer, pH 7.6; 5 μ moles NaCl and 170 μ moles sucrose. The complete reaction mixture also contained: 4 μ moles ATP; 10 μ moles PEP; 10 μ g PEP-kinase and about 2 mg of protein from the guinea pig liver 105,000 $\times g$ supernatant (cell sap). Mixture of radioactive amino acids was used as indicated in table 1; 130 μ g of nucleolar protein was used. After 10 minutes' incubation at 37 C, the nucleoli were centrifuged at 5,000 $\times g$ for 10 minutes and then washed twice by gentle resuspension in 0.88 M sucrose. One sample was extracted with 0.1 N HCl to separate the nucleolar basic-protein fraction from the residual-protein fraction, and the radioactivity of these fractions, as well as the radioactivity contained in the whole nucleoli, were estimated. The acid extract and the residual proteins contained 41 and 89 μ g of protein, respectively.

FRACTIONATION OF NUCLEAR AND NUCLEOLAR PROTEINS

Isolated intact nuclei were gently washed with 0.1 M potassium phosphate buffer, pH 7.1, to remove cytoplasmic contaminants and fragments of the nuclear envelope (1). The nuclei were then incubated with deoxyribonuclease at 37 C for 30–60 minutes; this step was necessary to remove the DNA and thus to make the extraction of nuclear proteins possible. After centrifugation, a supernatant was obtained which contained some of the nonbasic nuclear proteins. After dialysis, this extract was found DNA- and RNA-free. The residue was then extracted with 0.1 N HCl for 1 hour, which allows a complete extraction of the basic nuclear proteins. This fraction was DNA- and RNA-free. The residue was then submitted to extraction with 0.1 M Tris buffer, pH 7.3; this extract contains a very small percentage of nuclear proteins and about 60–65% of total nuclear RNA; the ratio of RNA to protein is often close to unity. This fraction may represent the nuclear ribosomal fraction (4). The final residue contains unextractable nuclear proteins plus 35–40% of nuclear RNA.

The same fractionation procedure was applied to the total nuclear material after sonication. In this case, centrifugation after deoxyribonuclease treatment was omitted because the solution did not need clarification.

The protein content of the nuclear fractions is reported in table 3. The first series of experiments shows the percentage of protein in each extract obtained from intact nuclei. The second series shows that after sonica-

TABLE 3.—Fractionation of nuclear and nucleolar proteins: percent of protein content of single fraction*

Series		Fraction	Protein (%)		
			Experi- ment 1	2	3
1st	Intact nuclei.....	0.1 M phosphate pH 7.1 extract...	22	25	16
		Deoxyribonuclease incubation extract.	12	17	14
		0.1 N HCl extract.....	36	30	38
		0.1 M Tris pH 7.3 extract.....	4	3	5
		Final residue.....	26	25	27
2d	Sonicated nuclei....	0.1 N HCl extract.....	78		
		Final residue.....	0.3		
3d	Nucleoli.....	0.1 N HCl extract.....	52	64	70
		Final residue.....	48	36	30

*The detailed description of the fractionation procedures is given in the text.

tion most of the nuclear proteins are no longer precipitable on 0.1 N HCl. The acid extract obtained from sonicated nuclei represents a heterogeneous protein fraction containing basic and nonbasic proteins, as described below. On the other hand, 52–70% of the total proteins of the nucleoli is soluble in 0.1 N HCl. The variability probably reflects nucleoplasmic contamination.

Fast Green Staining

The method developed by R. C. Huang [*see* (5)], which depends on the formation of a complex between the acid dye Fast Green and basic proteins on paper discs, has been applied to our preparations. Fifty and 100 μ g of each protein fraction were used and each experiment was run in duplicate. The amount of proteins was determined by the procedure of Lowry *et al.* (6).

The results presented in table 4 indicate that the amount of the Fast Green dye complexed with the basic proteins of intact nuclei is about twice as great as that complexed with proteins of sonicated nuclei. This observation may indicate that half of the proteins extracted by 0.1 N HCl from sonicated nuclei is nonbasic. This agrees with the data (*see* table 3) showing that the quantity of protein extracted from sonicated nuclei is about twice as much as that obtained from intact nuclei. The same observation was made in the nucleoli; these data may be explained by contamination with nucleoplasmic material.

TABLE 4.—Absorbancy of Fast Green complex with basic proteins of 0.1 N HCl extracts of nuclei and nucleoli

Sample	Absorbancy at 570 m μ /100 μ g protein	Total protein extracted by HCl (%)
Intact nuclei.....	0.380	36
Intact nuclei.....	0.440	34
Sonicated nuclei.....	0.180	78
Nucleoli.....	0.232	70
Calf thymus histone.....	0.440

Fifty and 100 μ g of protein [as measured by the procedure of Lowry *et al.* (6)] were dried on a Whatman 3 M filter paper disc and then immersed in a solution containing 0.1% Fast Green in water, pH 8.0, for 30 minutes at 0 C. The discs were washed with cold water, at pH 8.0, until all the unreacted dye was removed. They were dried, eluted with 1–2 ml of 0.3 N KOH, and then the absorbancy at 570 m μ was determined with a suitable blank. Calf thymus histones (Sigma) were used as a reference.

Disc Electrophoresis

Disc electrophoresis of the protein fractions extracted by acid pH was carried out on columns (8 \times 0.8 cm diameter) of 15% polyacrylamide gel (7). The electrophoresis was carried out at pH 4.3 (5 amp per tube, for 5 hours). The gels were fixed and stained with Amidoshwarz.

Figure 1 shows the electrophoresis of the basic proteins isolated from nuclei and nuclear subfractions. In the preparations of nuclei, five or sometimes more fast-moving bands and two major heavy bands can be distinguished. Difference in the electrophoretic patterns can be seen in the nuclear subfractions isolated after sonication, namely, nucleoplasmic fractions I and II and the nucleoli; the latter is probably contaminated by nucleoplasmic material. The main difference is the almost complete disappearance of the fast-moving bands (known to be the lysine-rich- α -histones). The middle heavy band (γ -histones) does not show any variation, whereas the first portion of the electrophoretic pattern is resolved into three or more bands, probably representing a better resolution of the first heavy band (β -histones).

These observations indicate that sonication damages the nuclear proteins. This damage may be held responsible for the inability of sonicated nuclei to carry out, *in vitro*, incorporation of amino acids into proteins.

Since criteria of purity of isolated nucleoli are not yet clearly established, it is difficult at the present to know whether the nucleoli are damaged in some way by sonication. However, that the nucleolar structure is somewhat preserved is indicated by the fact that the isolated nucleoli are active in incorporating amino acids.

RESUMEN

Se ha estudiado en núcleos y nucleolos de hígado de cobayo la capacidad de incorporar aminoácidos marcados en las proteínas en un sistema *in vitro*. Los resultados indican que mientras que la incorporación en los *núcleos* depende de la adición de cofactores, los *nucleolos*, no sólo no requieren estos cofactores, sino que son inhibidos por ellos. La actividad incorporante de los nucleolos aislados es mucho más elevada que la exhibida por los núcleos.

Se encontró que la incorporación marcada se distribuye en las fracciones proteicas nucleolares tanto *básicas* como no *básicas*, mostrando las primeras una elevada actividad específica.

Las proteínas básicas, extraídas de los núcleos y de fracciones subnucleares (nucleoplasma I y II y nucleolos) fueron analizadas mediante electroforesis de disco, para verificar si hubo algún daño proteico como resultado del procedimiento de aislación. En la electroforesis de las subfracciones nucleares, se observó una desaparición completa de las bandas de desplazamiento rápido, lo que sugiere que los ultrasonidos probablemente causaron algún daño.

REFERENCES

- (1) MAGGIO, R., SIEKEVITZ, P., and PALADE, G. E.: Studies on isolated nuclei. I. Isolation and chemical characterization of a nuclear fraction from guinea pig liver. *J Cell Biol* 18: 267-291, 1963.
- (2) ———: Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic subfractions. *J Cell Biol* 18: 293-312, 1963.
- (3) BIRNSTIEL, M. L., and HYDE, B. B.: Protein synthesis by isolated pea nucleoli. *J Cell Biol* 18: 41-50, 1963.
- (4) FRENSTER, J. H., ALLFREY, V. G., and MIRSKY, A. E.: Metabolism and morphology of ribonucleoprotein particles from the cell nucleus of lymphocytes. *Proc Nat Acad Sci USA* 46: 432-444, 1960.
- (5) BIRNSTIEL, M. L., CHIPCHASE, M. I., and FLAMM, W. G.: On the chemistry and organisation of nucleolar proteins. *Biochim Biophys Acta* 87: 111-122, 1964.
- (6) LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J.: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275, 1951.
- (7) REISFELD, R. A., LEWIS, U. F., and WILLIAMS, D. E.: Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature (London)* 195: 281-283, 1962.

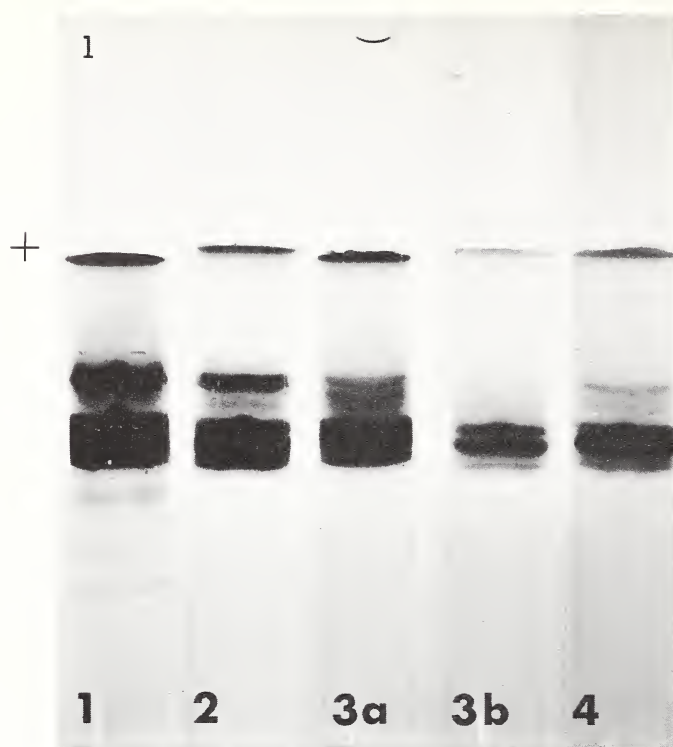


FIGURE 1.—Disc electrophoresis of basic proteins of nuclei and nucleolar subfractions. 1) Nuclei: 0.3 mg of protein. 2) Nucleoplasm fraction II: 0.25 mg of protein. 3) Nucleoplasm fraction I: (a) 0.55 mg of protein; (b) 0.3 mg of protein. 4) Nucleoli: 0.4 mg of protein.

DISCUSSION

Perry: Were the nucleoli that you demonstrated to possess amino acid incorporating activity prepared by sonication?

Maggio: Yes.

Perry: But sonicated whole nuclei had no activity. How does one explain this?

Maggio: The amount of nucleolar proteins is less than 10% of the total nuclear proteins and therefore the amount of nuclei used for the *in vitro* incorporating system may not be enough to demonstrate nucleolar incorporating activity, or there may be an inhibition by the sonicated nucleoplasmic material. The activity of the isolated nuclei is completely lost after sonication and this could mean that the activity is in some way linked to the structure of the nucleus.

Mandel: May I ask you two questions? First, you said complete medium with nucleoli. What is the complete medium?

Maggio: The complete reaction mixture contains ATP, an ATP regenerating system, plus cell sap. The nucleoli do not require these additional cofactors; therefore, nucleoli alone were incubated in the buffer plus sucrose without added ATP and cell sap. However, the complete system consisting of ATP, an ATP regenerating system, and cell sap was used with the nuclei.

Mandel: Now the second question. Have you any evidence about the nucleolar fraction into which the amino acids are incorporated? Is it the histone fraction or the residual protein?

Maggio: No. Unfortunately I have not been able to analyze them. The only thing I know is that the counts are distributed in both the acid-soluble and insoluble nucleolar protein fractions and that the former fraction has a higher specific activity.

Cohen: Did you do fingerprinting of any fraction to see whether the incorporation was in peptides or not?

Maggio: I tried to investigate this because it is important to know whether the incorporated amino acids were in peptide chains. I tried to apply Sanger's reaction, *i.e.*, the reaction with the fluorodinitrobenzene (as described by Fraenkel-Conrat *et al.*, in *Methods of Biochemical Analysis*, vol 2, p 359). Most of the radioactivity found after hydrolysis and ether extraction was in the aqueous phase. This could indicate that the incorporated counts were in peptide bonds but, since the hydrolysis was not complete, we were not able to conclude this with certainty.

Busch: In the sonicated nuclear preparation a large number of hydrolytic enzymes of the extranucleolar fraction are released from latent states and these enzymes hydrolyze existing soluble RNA, ribosomal RNA, and polysomal templates. However, the nucleolus still stays intact, and its components are undegraded. I think Dr. Maggio's experiment is quite intact on that point.

Vincent: What was the incubation time on the incorporation experiments?

Maggio: For the nucleoli it was very short, in this case between 5 and 10 minutes.

Vincent: I ask this question because with the starfish nucleoli a lag of 15 minutes before incorporation begins to occur at an appreciable rate, and this has also been demonstrated in other forms. For some reason, it takes some time for this reaction to go.

Maggio: The kinetics of guinea pig nucleolar incorporation is linear for the first 5 minutes and then reaches a plateau. This is why I used the first few minutes in order to be in the proportional parts of the curve. Sometimes we have observed a decrease of incorporated counts between 5 and 10 minutes, but we don't know the reason for this.

Vincent: We have found with the starfish that soluble RNA with the activated amino acid present apparently becomes bound to one of the protein constituents of

the nucleolus and moves with it in any type of purification procedure, including acid precipitation. But most of the counts that appeared to be incorporated were actually alkali labile and therefore had never gone into protein.

Birnstiel: I would like to come to the aid of Dr. Maggio. There is little doubt that isolated nucleoli can incorporate amino acids with kinetics very similar to those described in this paper. As evident from Dr. Maggio's presentation, the labeled amino acids are incorporated into hot-TCA-precipitable compounds, and this alone should exclude the possibility that the amino acids are bound solely to transfer RNA.

Perry: Dr. Maggio, the counts that referred to amino acid incorporation were resistant to hot TCA, weren't they? So that answers Dr. Vincent's question. One other comment: Have you, Dr. Maggio, or has anyone else, successfully isolated anything like a particle from a nucleolar preparation of animal cell origin? That is, can anyone obtain either subunits or whole ribosomes from isolated animal nucleoli?

Maggio: No, I have not isolated such particles.

Basilio: You mentioned that the intact nuclei require cell sap for amino acid incorporation. Do you know which component of the cell sap is actually required for the incorporation?

Maggio: No. The cell sap used was only a crude extract.

Composition of the Nucleolus as a Basis for Views on Its Function^{1,2}

JAN-ERIK EDSTRÖM, *Department of Histology, Karolinska Institutet, Stockholm 60, Sweden*

SUMMARY

Data on the composition of the nucleolus, mainly concerning RNA, are reviewed as a background for the presentation of ideas concerning its functional role. It is pointed out that the nature of the presumed synthetic products of the nucleolus cannot at present form a basis for a unifying concept of a specific function for this organelle. Diversity in biochemistry can, however, be compatible with a role for the nucleolus that is common to nucleated cells. The original suggestion by Vincent that the nucleolus protects nucleolar messenger RNA against ribonucleases could be extended

to include also other kinds of RNA for cytoplasmic export, transfer and ribosomal RNA. Work done in the author's laboratory on the RNA from the polytene genome of *Chironomus* is reviewed. Biochemical techniques have been adapted to the cellular level and data on the RNA of various components including nucleoli are given. It is shown that the use of density gradient sedimentation is also a possibility on this level and can contribute to a safer identification of the source of the synthetic products.—*Nat Cancer Inst Monogr* 23: 223–233, 1966.

KNOWLEDGE of the composition of the nucleolus is one of the foundations for the understanding of its function. Information on this subject has been obtained by a multitude of techniques—cytochemical and biochemical. This paper deals mainly with biochemical work, with emphasis on work on RNA.

THE RELATION OF THE NUCLEOLUS TO THE NUCLEIC ACIDS

Deoxyribonucleic Acid

While on the whole there has been agreement on the definition of nucleolar RNA, the definition of nucleolar DNA has been considerably less clear. Theoretically, several kinds of DNA could be considered. It

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported by grants from the Swedish Cancer Society.

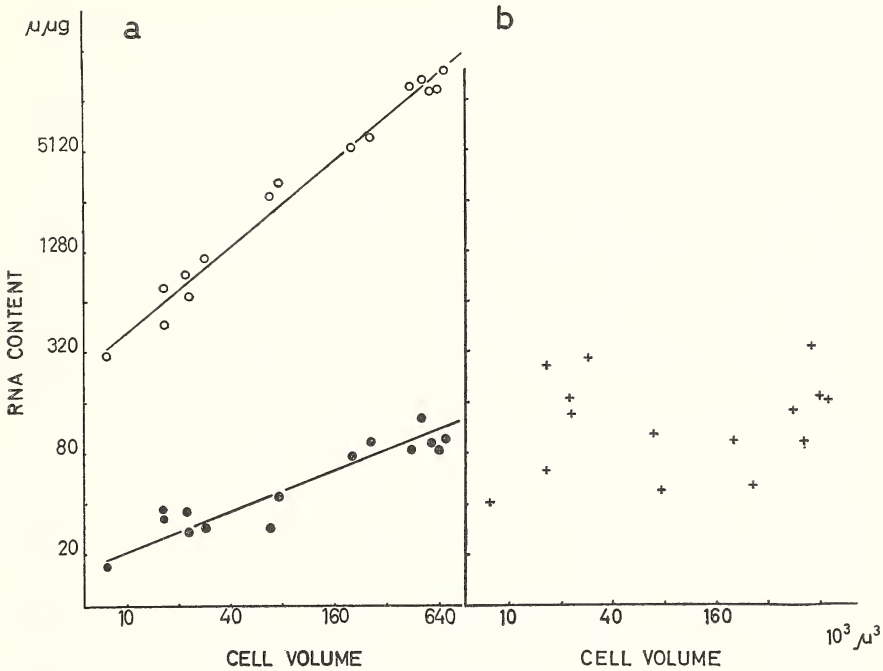
has been shown for *Drosophila* that DNA cistrons for RNA, of a kind known to be present in the nucleolus, are present in the part of the chromosome around which the nucleolus is synthesized (1). In polytene chromosomes this part can be compared to a more or less unfolded chromosome band, whose strands branch in the center of the nucleolus or extend into its matrix (2-4). Furthermore, it has been shown that these chromosomal regions are necessary for the development of the nucleolus in *Chironomus* (4). This part of the genome is usually called the nucleolar organizer (NO), although its role may differ from that of certain plant cell nucleoli, in which the part of the genome responsible for the formation of the nucleolus has been denoted NO on the basis of its presumed action, *viz*, collecting and organizing nucleolar material produced elsewhere. These kinds of DNA are, as far as is known, not synthesized with nucleolar RNA but are part of the constant chromosome set. In contrast there is DNA, probably specifically developed with the nucleoli in amphibian oocyte peripheral nucleoli, where in all likelihood it is responsible for the synthesis of the nucleolar RNA (5).

These three kinds of DNA could be considered as nucleolar components, and they all probably constitute a relatively small fraction of the total nucleolar mass, at least in well-developed nucleoli. The DNA surrounding nucleoli, the nucleolus-associated chromatin, on the other hand, might or might not be functionally related specifically to the nucleolus. As long as the possibility cannot be excluded, however, that its association to the nucleolus is caused by random factors, it has to be considered separately from the nucleolus. Except in preparations of starfish nucleoli, there seems to be considerable DNA present in nucleoli isolated in bulk. Its presence could be responsible for the high DNA content of several preparations and the reported effects of histones on the composition of RNA synthesized by isolated nucleoli (6).

Ribonucleic Acid

Relationship of Nucleolar and Cytoplasmic RNA

In growing oocytes both nucleolar and cytoplasmic RNA content are well correlated with cell size (7). Non-nucleolar nuclear RNA, on the other hand, varies irregularly between cells and is independent of cell size (text-fig. 1). Also in secretory cells (8), at different activities and varying cell size and RNA content, nucleolar size and RNA content are correlated with the cellular (mainly cytoplasmic) RNA. No correlation in amounts or composition between nucleolar and other nuclear RNA has been observed; in fact, cells with most of the protein synthesis located in the nuclei, such as thymocytes (9), have poorly developed nucleoli. This raises the question whether the development of the nucleolus is caused by the creation during phylogenetic development of protein synthesis in a separated cytoplasm rather than by a specific need for an organelle of



TEXT-FIGURE 1.—Cytoplasmic and nucleolar RNA (a) and nucleoplasmic RNA (b) plotted against cell size in growing starfish oocytes. \circ : Cytoplasmic RNA. \bullet : Nucleolar RNA.

this kind for the purpose of ribosomal RNA synthesis. Perhaps this is why it is absent in the anucleate or (more properly) acytoplasmic bacteria.

There is also other evidence, based on the composition of the nucleolus, that stresses the relationship between cytoplasmic and nucleolar RNA specifically among the nuclear RNA fractions. In microelectrophoretic analyses (10) the nucleolar RNA has been found, alone among the nuclear fractions, to resemble cytoplasmic RNA, at least in animal cells (table 1). This may not always be true, since the nucleoli in mature starfish oocytes were found to contain RNA appreciably different in composition from that of the cytoplasmic RNA (11). These nucleoli were about to be dissolved, however, and the RNA in nucleoli of young oocytes had a base composition indistinguishable from that of the cytoplasm.

Nucleolar 4S RNA

The use of unfractionated RNA for base analysis gives information of limited value, of course, unless the source is homogeneous. Vincent found two fractions in nucleolar RNA from starfish oocytes (12), one with a rapid turnover and a second more stable one. He has confirmed and extended these observations in later work (13, 14). The two kinds of RNA have been identified as ribosomal and 4S RNA, respectively.

TABLE 1.—Composition of RNA from nucleolus and cytoplasm, and DNA composition from various species

	Adenine	Guanine	Cyto- sine	Uracil	Guanine + Cytosine
<i>Triturus cristatus</i> , oocyte					
Nucleoli.....	18.1	31.7	28.7	21.7	60.4
Cytoplasm.....	20.1	27.2	29.5	23.0	56.7
DNA.....	27.5	22.5	—	—	45.0
<i>Triturus viridescens</i> , oocyte					
Nucleoli.....	21.5	29.3	30.1	19.1	59.4
Cytoplasm.....	23.7	28.0	27.7	20.7	55.7
DNA.....	27.3	22.7	—	—	45.4
<i>Asterias</i> , oocyte					
Nucleoli.....	23.7	33.4	24.3	18.5	57.7
Cytoplasm.....	23.5	31.9	24.8	19.7	56.7
DNA*.....	36.0	14.0	—	—	28.0
<i>Tegenaria</i> , oocyte					
Nucleoli.....	25.2	29.8	22.9	22.2	52.7
Cytoplasm.....	25.1	30.2	21.9	22.9	52.1
<i>Chironomus</i> , salivary gland					
Nucleoli.....	30.6	20.1	22.1	27.1	42.2
Cytoplasm.....	29.4	22.9	22.1	25.7	45.0
DNA.....	35.1	14.9	—	—	29.8
<i>Drosophila</i> ,† salivary gland					
Nucleoli.....	34.9	21.7	19.1	24.2	40.8
Ribosomes.....	32.5	20.8	19.4	27.3	40.2
DNA.....	29.6	20.4	—	—	40.8
<i>Acetabularia</i> ,‡					
Nucleoli.....	31.3	20.3	17.9	30.6	38.2
Cytoplasm.....	25.6	25.6	23.4	25.5	49.0
DNA.....	29.0	21.0	—	—	42.0

*Quoted from Vincent (14).

†Unpublished analyses by the author on material provided by Dr. F. M. Ritossa.

‡Unpublished work by R. Tencer, E. Baltus, and J.-E. Edström.

The nucleolar 4S RNA has been found to possess characteristics of transfer RNA, such as becoming methylated at the macromolecular level (15-17), pseudouridine incorporation (18), terminal incorporation of adenine and cytosine (13), and incorporation of leucine or lysine into alkali labile bonds (14). When chromosomal RNA synthesis was inhibited in *Smittia* by substituted benzimidazoles, 4S synthesis of transfer RNA character still occurred (17). In other cells, enucleolated by pharmacological or genetic means, 4S synthesis was, on the other hand, still observable (19, 20), although in these cases an identification of 4S RNA with transfer RNA was not made.

Nucleolar Ribosomal RNA

In addition to the indirect evidence for the presence of ribosomal RNA in the nucleolus provided by the quoted base analyses, direct demonstrations of ribosomes and ribosomal RNA components have been made in isolated nucleolar preparations (21). In apparent agreement with this, the anucleolate mutants of *Xenopus laevis* are unable to provide ribosomal RNA (19), a result that would occur if ribosomal RNA is synthesized in the NO. In this instance, however, other explanations may be suggested. As pointed out by these authors, an operator mutation may be involved.

Another possibility is that a normal-functioning nucleolus provides a factor that stabilizes intranuclear RNA delivered here (14). If unstable, the ribosomal RNA components might not be detectable in a large pool of other, rapidly turning-over RNA. Perry (20) found that low concentrations of actinomycin inhibited incorporations of RNA precursors into the nucleolus and that under such conditions high molecular precursors to ribosomal RNA were no longer formed. Whether these precursors are formed and rapidly broken down or whether all synthesis is inhibited, the results nonetheless show that the nucleolus contains precursors of ribosomal RNA; nucleolar origin is likely but not proved. In a later paper Perry *et al.* (22) showed that the supposed precursors and ribosomal components compete for the same DNA cistrons, indicating identity of source.

Other recent evidence identifies more conclusively the origin of ribosomal RNA with the NO. In a preliminary report, Birnstiel and Wallace (23) indicated that the DNA templates for ribosomal RNA synthesis have their localization in the NO region of *Xenopus laevis*. Furthermore DNA templates for ribosomal RNA were enriched in HeLa cell nucleoli (24). Ritossa and Spiegelman (1), finally, showed the exclusive localization of DNA templates for ribosomal RNA in the NO region of *Drosophila*. Strong evidence for a local origin of at least some nucleolar RNA had earlier been obtained by Pelling (25).

In plants, however, the available evidence is more for a disperse than a local origin of nucleolar material in general. According to Heitz (26), the size of a nucleolus is dependent on the amount of chromatin also in the absence of an organizer in partial nuclei isolated from the rest of the nucleus. In other instances, in the absence of a functional NO, drop-lets of nucleolar-like material accumulate along the chromosomes (27). Rho and Bonner (28), in incorporation and chase experiments, obtained results indicating that the chromatin is the primary site of synthesis of nucleolar RNA in pea nuclei. Chipchase and Birnstiel (29) found that cistrons for ribosomal RNA synthesis are not enriched in the DNA isolated with the nucleoli from the same material.

Since evidence from plants suggests that a localization of ribosomal RNA cistrons to the NO region may not be universal, one should be cautious when judging the same situation in animal cells, and perhaps avoid making a general conclusion from evidence obtained from different species of animals. Except for the preliminary report on the localization of ribosomal RNA cistrons in the NO of *Xenopus*, there is only one instance (in *Drosophila*) in which the exclusive localization of cistrons for ribosomal RNA to the NO's has been demonstrated.

Nucleolar Messenger RNA

The idea that the nucleolus has a function in stabilizing a hypothetical nucleolar messenger RNA has recently been advanced by Vincent (14) on the basis of his findings of a lysine-rich, intranucleolar protein capable

of inactivating ribonuclease. This theory could be extended to include not only messenger RNA but also other types of RNA, perhaps in the first instance ribosomal RNA. The intranuclear breakdown of DNA-like rapidly labeled RNA, reported by some workers (30, 31), does not normally seem to affect ribosomal RNA but does so when actinomycin is given, *i.e.*, nucleolar synthetic functions are interfered with before a certain critical time after synthesis of ribosomal RNA precursors (32). As pointed out previously, some results might profitably be re-evaluated with this hypothesis in mind.

On the other hand, neither the formation nor the survival of 4S RNA seems to depend on the presence of a functioning nucleolus (19, 20). This could perhaps be an argument against the idea that the nucleolus has a general protective influence against RNA degradation; perhaps complementarity or other physical reasons may be of importance here. It should also be remembered that 4S RNA may represent other RNA than transfer RNA (33). It is an often-observed phenomenon that 4S material is a degradation product of high molecular material. Therefore the results on non-nucleolar localization of 4S RNA synthesis may not be contradictory to those of Birnstiel *et al.* (17).

If the specific role of the nucleolus is to prevent breakdown of RNA meant for export to the cytoplasm, it might be worthwhile to look for an association of messenger RNA also with the nucleolus. With messenger RNA, the problem is complicated by the fact that the ideas about its origin are far from clear in nucleated cells. By definition, it has to be formed in the genome, *i.e.*, on the chromosomes, but it is not known what part of the chromosomal RNA is messenger RNA, and what part never enters the cytoplasm, and whether the latter part is nonfunctional in an informational sense and is broken down. In any event, messenger RNA has to escape degradation due to intranuclear ribonucleases, and it is an interesting question whether the nucleolus may protect such RNA from breakdown. Perhaps pictures of chromatin associated with nucleoli could be explained in this way. Of interest in this connection is the work by McConkey and Hopkins (34) and others, suggesting a formation already in the nucleus of a close association between messenger RNA and a light ribosomal component (45S).

EXPERIMENTAL USE OF THE POLYTENE GENOME IN THE STUDY OF NUCLEOLAR RNA

Methods and Materials

A material, ideal in many respects, is provided by the salivary glands of the dipteran, *Chironomus tentans*. By micromanipulation, nuclear components such as individual polytene chromosomes, small chromosome

segments, nucleoli, and nuclear sap can be isolated (35). Microdissection of individual cellular units does not provide a large amount of material even when giant cells or giant chromosomes are used. By microelectrophoresis it is, however, possible to handle amounts on the scale of the single cell and determine the amount of the two nucleic acids and their base composition (36, 37). Furthermore, on the basis of P^{32} -labeling, the apparent base composition can also be assessed according to Volkin and Astrachan (38) and finally, with P^{32} again, it is possible in these dimensions to study the molecular distribution of RNA isolated from components in microscopic quantities by sedimentation analysis. It seems likely that a direct electrophoretic analysis of macromolecular RNA distribution in an extract will also become a possibility. This is currently being investigated in our laboratory. Perhaps the most fascinating feature of the system is that it permits the isolation for analysis of nucleoli from different organizers as well as individual puffed bands (such segments include more than one band, but practically all RNA originates from a single band).

Results

Base Composition Studies

The base composition of RNA and DNA determined for various components of the genome of *Chironomus tentans* is given in table 2. On the whole, one is struck by the high adenine content of DNA, unusual for a nucleated organism. It is not a characteristic trait for Diptera, since *Drosophila* has a more typical DNA (39).

On the whole, RNA, which is also adenine-rich, differs from DNA in containing a much higher G + C (guanine + cytosine) content, whether determined by microelectrophoresis or P^{32} base analysis. In view of the unimodal and typically narrow distribution of DNA when sedimented in the cesium chloride gradient (40), the conclusion has to be drawn that either a very unrepresentative DNA is synthetically active or RNA is secondarily modified. For the nucleolus, the differences in G + C content between RNA and DNA amount to 11% when RNA is analyzed by microelectrophoresis and 14% when it is done with P^{32} . In *Drosophila*, on the other hand, the agreement in G + C content is very good between DNA and chromosomal as well as nucleolar RNA (table 2).

Base analysis disclosed distinct differences between the properties of RNA from nucleoli and those of the chromosomes. Nucleolar RNA from the NO's of the second and third chromosome has, within narrow margins, the same base composition (41), while RNA from different non-nucleolar chromosomal segments (35) shows significant variations (table 2). This is, of course, in accord with the notion that the nucleoli are functionally equivalent (4) and the chromosome bands functionally different.

TABLE 2.—Base composition of RNA and DNA from nuclear components of salivary gland cells

	<i>Chironomus</i>						<i>Drosophila</i> *		
	RNA			DNA			RNA		DNA
	Polytene chromosomes			Nucleoli			Polytene chromo- somes	Nucleoli	Polytene chromo- somes
	Microelectro- phoresis		p ₂₂	Microelectro- phoresis		p ₂₂			
	Chrom. I	Balbani ring	Whole set	Nuel. II	Nuel. III	Nuel. combined			
Adenine.....	29.4	38.0	33.1	31.0	30.8	30.3	40.7	34.9	29.6
Guanine.....	19.8	20.5	19.3	22.8	22.2	22.7	23.2	21.7	20.4
Cytosine.....	27.7	24.5	21.3	18.5	18.8	21.5	19.1	19.1	—
Uracil.....	23.1	17.1	26.4	27.7	28.3	25.6	17.0	24.2	—
Guanine + cytosine.....	47.5	45.0	40.6	41.3	41.0	44.2	42.3	40.8	40.8

*Unpublished microelectrophoretic analyses performed by the author on material provided by Dr. F. M. Ritossa.

CONCLUSION

The object of this paper has been to present, without any claims to originality, ideas which, correct or not, provide the background and the stimulus to the work currently being performed on the giant genome of *Chironomus*. I have tried to show that it is hopeful the integrated study of the nuclear components of these nuclei will offer specific advantages in the analysis of their composition and function.

RESUMEN

Se reveen datos sobre la composición del nucleolo, principalmente en lo que se refiere al ARN, como fundamento de una presentación de ideas acerca de su papel funcional. Se señala que la naturaleza de los supuestos productos sintéticos del nucleolo no puede, en la actualidad, formar la base de un concepto unificador de un papel específico de este organelo. La diversidad bioquímica puede, sin embargo, ser no menos compatible con un papel para el nucleolo común a las células nucleadas. La original sugerencia de Vincent de que este papel es la protección del ARN mensajero nucleolar contra las ribonucleasas pudiera extenderse o incluir también otras clases de ARN de exportación citoplásmica, ARN de transferencia y ribosómico. Se analizó la labor realizada en el laboratorio del autor sobre el ARN del genomio politénico de *Chironomus*. Se han adaptado técnicas bioquímicas a nivel celular y se dan datos sobre los ARN de varios componentes inclusive el nucleolo. Se muestra que el gradiente de densidad de sedimentación es también, a este nivel, una posibilidad y puede contribuir a una identificación más segura del origen de los productos sintéticos.

REFERENCES

- (1) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. Proc Nat Acad Sci USA 53: 737-745, 1965.
- (2) KALNINS, V. I., STICH, H. F., and BENCOSME, S. A.: Fine structure of the nucleolar organizer of salivary gland chromosome of *Chironomids*. J Ultrastruct Res 11: 282-291, 1964.
- (3) STEVENS, B. J.: The effect of actinomycin D on nucleolar and nuclear fine structure in the salivary gland cell of *Chironomus thummi*. J Ultrastruct Res 11: 329-353, 1964.
- (4) BEERMANN, W.: Der Nukleolus als Lebenswichtiger Bestandteil des Zellkernes. Chromosoma 11: 263-296, 1960.
- (5) MILLER, O. L.: Extrachromosomal nucleolar DNA in amphibian oocytes. J Cell Biol 23: 60A, 1964.
- (6) LIAU, M. C., HNILICA, L. S., and HURLBERT, R. B.: Regulation of RNA synthesis in isolated nucleoli by histones and nucleolar proteins. Proc Nat Acad Sci USA 53: 626-633, 1965.
- (7) EDSTRÖM, J.-E., GRAMPP, W., and SCHOR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. J Biophys Biochem Cytol 11: 549-557, 1961.
- (8) EDSTRÖM, J.-E., and EICHNER, D.: Quantitative Ribonukleinsäure-Untersuchungen an den Ganglienzellen des Nucleus Supraopticus der Albino-Ratte unter

- Experimentellen Bedingungen (Kochsalz-Belastung). *Z Zellforsch* 48: 187-200, 1958.
- (9) FRENSTER, J. H., ALLFREY, V. G., and MIRSKY, A. E.: *In vitro* incorporation of amino acids into the proteins of isolated nuclear ribosomes. *Biochim Biophys Acta* 47: 130-137, 1961.
 - (10) EDSTRÖM, J.-E.: Chromosomal RNA and other nuclear RNA fractions. *In The Role of Chromosomes in Development* (Locke, M., ed.). New York and London, Academic Press Inc., 1964, pp 137-152.
 - (11) ———: Nucleolar RNA composition during growth of the starfish oocyte. *Biochim Biophys Res Commun* 18: 595-599, 1965.
 - (12) VINCENT, W. S.: Heterogeneity of nuclear ribonucleic acid. *Science* 126: 306-307, 1957.
 - (13) VINCENT, W. S., and BALTUS, E.: The ribonucleic acids of nucleoli. *In The Cell Nucleus* (Mitchell, J. S., ed.). New York and London, Academic Press Inc., 1960, pp 18-23.
 - (14) VINCENT, W. S.: The Nucleolus. *Proc XI Inter Congr Gen.* The Hague, Pergamon Press, 1964, pp 343-358.
 - (15) BIRNSTIEL, M. L., FLEISSNER, E., and BOREK, E.: Nucleolus: A center of RNA methylation. *Science* 142: 1577-1580, 1963.
 - (16) SIRLIN, J. L., JACOB, J., and TANDLER, C. J.: Transfer of the methyl group of methionine to nucleolar ribonucleic acid. *Biochem J* 89: 447-452, 1963.
 - (17) BIRNSTIEL, M. L., JACOB, J., and SIRLIN, J. L.: Analysis of nucleolar RNA synthesis in dipteran salivary glands. *Symposium on Molecular Cytology. In Arch Biol (Liege).* In press.
 - (18) SIRLIN, J. L., KATO, K., and JONES, K. W.: Synthesis of ribonucleic acid in the nucleolus. *Biochim Biophys Acta* 48: 421-423, 1961.
 - (19) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
 - (20) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
 - (21) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosomes. *Biochim Biophys Acta* 76: 454-462, 1963.
 - (22) PERRY, R. P., SRINIVASAN, P. R., and KELLEY, D. E.: Hybridization of rapidly labeled nuclear ribonucleic acids. *Science* 145: 504-507, 1964.
 - (23) BIRNSTIEL, M., and WALLACE, H.: Ribosomal RNA-DNA hybrids from *Xenopus laevis*. Abstract 2d meeting Fed Europ Biochem Soc Vienna, 1965, p 332.
 - (24) MCCONKEY, E. H., and HOPKINS, J. W.: The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 51: 1197-1204, 1964.
 - (25) PELLING, C.: Ribonukleinsäure-Synthese der Riesenchromosomen. *Chromosoma* 15: 71-122, 1964.
 - (26) HEITZ, E.: Nukleolen und Chromosomen in der Gattung *Vicia*. *Planta Med* 15: 495-505, 1931.
 - (27) MCCLINTOCK, B.: The relation of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
 - (28) RHO, J. H., and BONNER, J.: The site of ribonucleic acid synthesis in the isolated nucleus. *Proc Nat Acad Sci USA* 47: 1611-1619, 1961.
 - (29) CHIPCHASE, M. I. H., and BIRNSTIEL, M. L.: On the nature of nucleolar RNA. *Proc Nat Acad Sci USA* 50: 1101-1107, 1963.
 - (30) HARRIS, H., and WATTS, J. W.: The relationship between nuclear and cytoplasmic ribonucleic acid. *Proc Roy Soc (Biol)* 156: 109-121, 1962.
 - (31) BRUNS, G. P., FISCHER, S., and LOWY, B. A.: A study on the synthesis and interrelationships of ribonucleic acids in duck erythrocytes. *Biochim Biophys Acta* 95: 280-290, 1965.

- (32) GIRARD, M., PENMAN, S., and DARNELL, J. E.: The effect of actinomycin on ribosome formation in HeLa cells. *Proc Nat Acad Sci USA* 51:205-211, 1964.
- (33) GREENMAN, D. L., KENNEY, F. T., and WICKS, W. D.: On equating low molecular weight RNA with transfer RNA. *Biochem Biophys Res Commun* 17:449-453, 1965.
- (34) McCONKEY, E. H., and HOPKINS, J. W.: Subribosomal particles and the transport of messenger RNA in HeLa cells. *J Molec Biol* 14:257-270, 1965.
- (35) EDSTRÖM, J.-E., and BEERMANN, W.: The base composition of nucleic acids in chromosomes, puffs, nucleoli, and cytoplasm of *Chironomus* salivary gland cells. *J Cell Biol* 14:371-380, 1962.
- (36) EDSTRÖM, J.-E.: Microelectrophoretic determination of deoxyribonucleic acid content and base composition in microscopic tissue samples. *Biochim Biophys Acta* 80:399-410, 1964.
- (37) ———: Microextraction and microelectrophoresis for determination and analysis of nucleic acids in isolated cellular units. *In Methods in Cell Physiology* 1:417-447, 1964.
- (38) VOLKIN, E., and ASTRACHAN, L.: Phosphorus incorporation in *Escherichia coli* ribonucleic acid after infection with bacteriophage T2. *Virology* 2:149-161, 1956.
- (39) MEAD, C. G.: A deoxyribonucleic acid-associated ribonucleic acid from *Drosophila melanogaster*. *J Biol Chem* 239:550-554, 1964.
- (40) DAWID, I.: Personal communication, 1965.
- (41) EDSTRÖM, J.-E.: The base composition of RNA from nucleoli formed at two different organizers in *Chironomus tentans*. *Biochem Biophys Res Commun* 18:341-344, 1965.
- (42) BASSELL, B., and EDSTRÖM, J.-E.: To be published.

Proteins and Nucleic Acids of Starfish Oocyte Nucleoli and Ribosomes^{1, 2}

W. S. VINCENT, *Department of Anatomy and Cell Biology, University of Pittsburgh, Pittsburgh, Pennsylvania*; ELYANE BALTUS,³ *Laboratory of Animal Morphology, Free University of Brussels, Brussels, Belgium*; ARNE LØVLIE,⁴ *Zoological Laboratories, University of Oslo, Oslo, Norway*; and R. E. MUNDELL,⁵ *Department of Anatomy, Tufts University, Boston, Massachusetts*

SUMMARY

Protein and nucleic acid analyses of material from the starfish *Asterias forbesii* do not support the concept that the oocyte nucleolus is the site of significant storage of intact ribosomes, or the site of storage of 18S ribosomal RNA, or the site of storage of the spectrum of ribosomal proteins. Antigenic similarities between ribosomal and nucleolar proteins, confirmed by gel electrophoresis, do sug-

gest that some common proteins exist. The nucleolar role in ribosome biogenesis in this material would seem to be limited to 28S RNA plus the provision of certain proteins. This might be in the form of a ribosomal precursor RNP particle similar to the chloramphenicol particle found in bacterial systems.—*Nat Cancer Inst Monogr* 23: 235–253, 1966.

THE POSSIBILITY that the nucleolus is the site of ribosome synthesis and/or assembly is a major subject of this Symposium. If this possibility can be confirmed, the many frustrating years of study and description of the nucleolus would come to fruition in the demonstration of a specific nucleolar function (1–3). As the constituents of ribosomes are now well described in terms of morphology, chemical analysis, and particularly their RNA and protein content (4), nucleoli can be examined in a similar fashion and compared with ribosomes.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported in part by Public Health Service grant GM-11840 from the National Institute of General Medical Sciences and grant GB-3273 from the National Science Foundation. Portions of this work were done at the Marine Biological Laboratory, Woods Hole, Mass.

³ Fulbright Travel Grant recipient.

⁴ Research Fellow, Fonds National de la Recherche Scientifique, Belgique.

⁵ Supported in part by Public Health Service training grant HD-40-09 from the National Institute of Child Health and Human Development.

In this paper we report the results of such a comparison of nucleoli and ribosomes from the oocyte of the starfish, *Asterias forbesii*. The oocyte is the site of massive ribosome synthesis and storage; these ribosomes are later used during the development of the embryo (5). The oocytes contain very large, single nucleoli which can be isolated in large quantities (6, 7). The nucleoli contain 5% RNA and 95% protein. In previous reports we have demonstrated that the nucleoli of these oocytes will incorporate precursors into at least 2 RNA fractions (8), that one of these fractions has the characteristics of transfer RNA (9), and that the other has a base ratio identical with ribosomal RNA (10). We have reported also that this nucleolus is composed primarily of protein of a neutral or slightly acidic nature (6). This protein is ultracentrifugally homogeneous and reveals only a single end group upon DNFB analysis (10, 11). Approximately 85% of the dry weight of the nucleolus is this protein, which we have designated L-protein because of its high lysine content (10). The remaining 15% of the nucleolus is composed of basic proteins in association with a ribosomal-like RNA.

In the experiments reported we give evidence leading to the conclusions that: (a) certain proteins with common antigenic properties are found in starfish nucleoli and ribosomes; (b) certain basic proteins of the nucleolus appear to migrate in common with ribosomal proteins in disc electrophoresis; (c) many of the nucleolar basic proteins are dissimilar to ribosomal proteins; (d) the ribosomes contain many proteins not found in the nucleolus; (e) most of the protein contained in the nucleolus is unrelated to ribosomes; (f) nucleolar RNA contains diverse populations of molecules, most of which are 28–30S.

MATERIALS AND METHODS

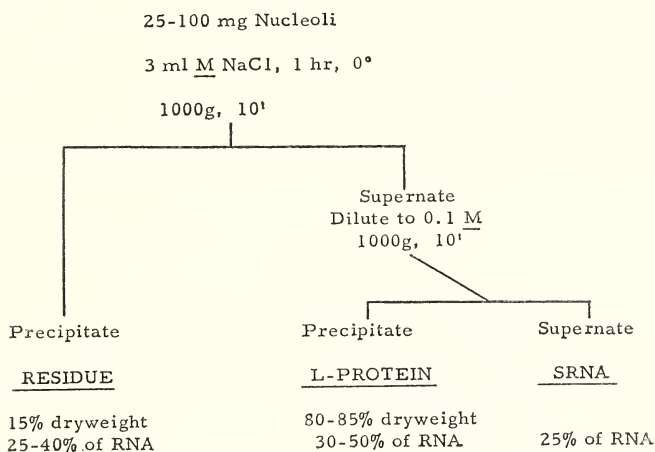
Isolation of nucleoli and ribosomes.—Nucleoli were isolated from *Asterias forbesii* oocytes according to Vincent (8, 10) except that the 1.8×10^{-4} M CaCl_2 was increased to 3.6×10^{-4} M in experiments involving nucleolar RNA sucrose gradient analysis and disc electrophoresis of nucleolar proteins.

Nucleoli were fractionated according to the scheme shown in text-figure 1. The various subfractions designated in the text: L-protein, residue, and S RNA were obtained as indicated.

Ribosomes were isolated from the supernatant of the nucleolar isolation as follows: The supernatant was centrifuged for 20 minutes at $10,000 \times g$, the supernatant fluid was then made to 1% deoxycholate and centrifuged for 1 hour at $105,000 \times g$. The pellet from this centrifugation was then resuspended with a glass homogenizer, centrifuged at $2,000 \times g$ for 10 minutes, and the supernatant suspension of ribosomes used where indicated.

Radioisotopes.—When isotopically labeled materials were used, labeled fractions were collected on Millipore filters, washed 3 times with cold 5%

FRACTIONATION OF NUCLEOLI



TEXT-FIGURE 1.—Fractionation of nucleoli.

trichloroacetic acid, 3 times with H_2O , and finally 3 times with 95% ethanol, and counted in a thin-window gas flow counter to $\pm 5\%$.

Preparation of RNA.—RNA was extracted as indicated in the text-figure legends. Centrifugation was carried out at 0–4°C in the SW25 Spinco head at 24,000 rpm for 40–45 hours. Exponential gradients were constructed of 10–40% sucrose in 0.005 M Tris, pH 7.4. Tracings of RNA distribution in the gradients were obtained by means of a flow cell and recording spectrophotometer. When RNA was not previously extracted, the ribosomal or nucleolar preparations were stirred for 1 hour at room temperature in 0.5% sodium dodecyl sulfate (SDS) and applied directly onto the gradient.

Preparation of antigens.—It has been established that antibody to one type of ribonucleoprotein will indiscriminately react with any other form of RNP (12, 13). As ribosomal and nucleolar proteins are typically associated with RNA, all RNA had to be removed from the preparations to be used as antigens. We have used the procedure described by Bigley *et al.* (14) to insure that nonspecific responses due to RNA were not complicating the serological results. Control tests against RNA were made in every experiment and were uniformly negative with the above procedure. Descriptions of the testing procedures used are found in the text-figure legends. The thickness of the skin response in the skin test series was measured according to Trakatellis *et al.* (15). All intracutaneous injections were 0.1 ml in volume.

Disc electrophoresis.—Gel electrophoresis was carried out on 7.5 or 15% gels made up at pH 5.4 in 8 M urea and run for approximately 2 hours at room temperature at 6 ma per tube according to the procedures of Reisfeld *et al.* (16). Staining and destaining were carried out as described by Leboy *et al.* (17). Ribosomal proteins were extracted with 1 M NaCl or

2 M LiCl at room temperature for 2–4 hours with stirring and with 66% acetic acid in the cold. They were dialyzed against 8 M urea and placed in sample gels in amounts ranging from 20–750 μ g. Nucleolar proteins were extracted by the same procedure, except for residue protein which was obtained from the residue fraction by 2 M LiCl. No significant difference in band patterns was observed between the NaCl, LiCl, or acetic acid extracted proteins except that LiCl extracts gave sharper bands.

RESULTS

Nucleolar and Ribosomal RNA

The results of phenol extraction of nucleolar RNA are variable when pH and temperature are changed (*A*, *A*₁), with no clear pattern of RNA subfractions emerging (text-fig. 2).

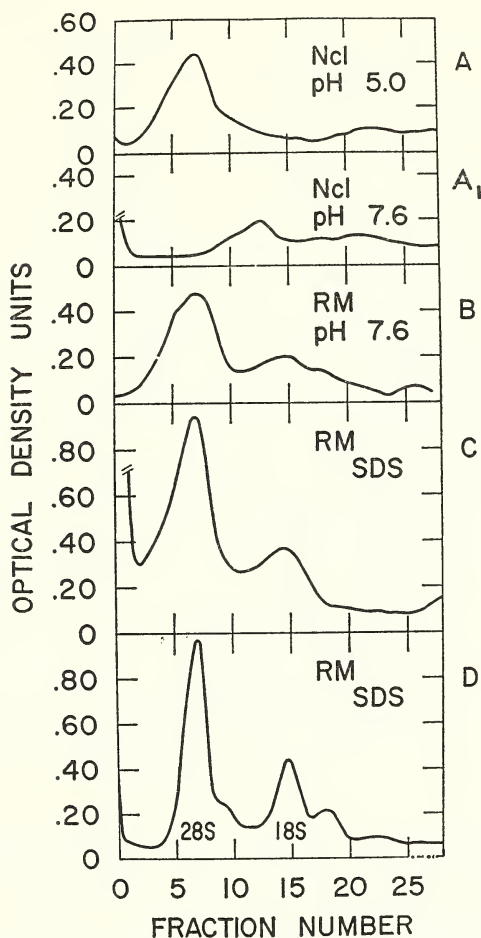
The result of phenol extraction of ribosomes at pH 7.6 (*2B*) is similar to the result obtained by the SDS extract (*2C*) of freshly prepared ribosomes, but with more diffuse peaks. SDS treatment of lyophilized ribosomes (*2D*) gives very sharp separation, with minor shoulders of breakdown products on each of the two major peaks. Similar treatment of lyophilized (text-fig. *3A*, *B*, *C*,) and fresh (text-fig. *3D*) nucleoli demonstrates sharp separation of RNA components.

Notable in all these preparations is that the heavy “28S” region shows two peaks differing by 3–5 S units. These vary in different nucleolar preparations; the significance of this variation and of the two peaks is not yet understood. In every preparation there is diffuse absorbance around fraction 15, the “18S” region. Whether this is 18S RNA or breakdown products of the large RNA fraction is not yet determined. There is no doubt that the RNA in these nucleoli, as has been found in other forms (*18–20*), is predominantly 28S or larger.

Ribosomal and Nucleolar Antigens

Nucleolar fractions were tested against ribosomal proteins for the presence of common antigens. We did a series of studies by testing for skin reactions to a test antigen after inoculation of a sensitizing antigen 1 week previously. The results of tests of the cross-reactions of ribosomal and nucleolar proteins are shown in text-figure 4. Each bar on the graph represents the difference between the response obtained with saline and the response obtained with the indicated test antigen after appropriate sensitization. The data were obtained from measurement of the response obtained at 3 separate sites on each of 5 to 8 animals.

L-protein was found to react primarily with itself, with variable response from RS + RM protein ($t = >0.1$). RM protein and RS protein respond



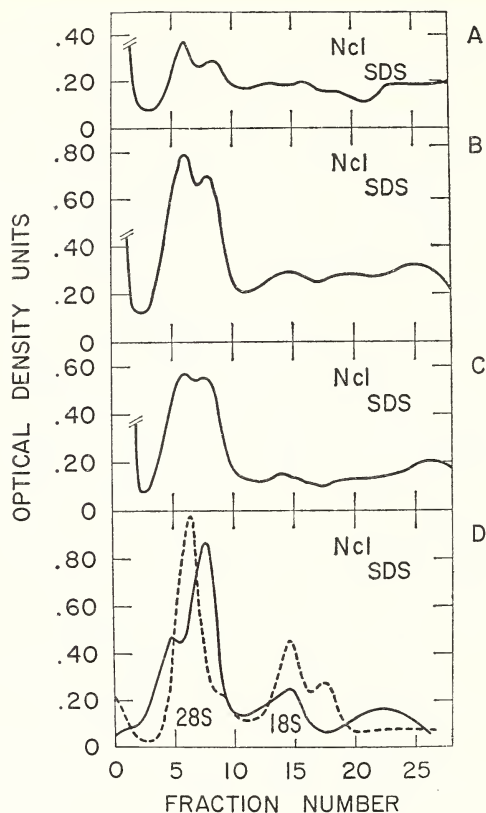
TEXT-FIGURE 2.—Gradient profiles of nucleolar and ribosomal RNA.

A: RNA extracted from freshly prepared nucleoli in 1.0 ml 0.1 M acetate buffer, pH 5.0, containing 1% SDS and 4 μ g polyvinylsulfate (PVS) plus 1 ml phenol containing 0.1% hydroxyquinoline for 10 minutes at 4 C.

*A*₁ and *B*: RNA extracted from freshly prepared nucleoli (*A*₁) and ribosomes (*B*) in 1.5 ml 0.05 M Tris buffer, pH 7.6, containing 4 μ g PVS/ml plus 1.5 ml phenol for 10 minutes at 60 C. Supernatant aqueous layer was made to 1 M with NaCl and the RNA precipitated with 2 volumes of ethanol at -20 C overnight.

C and *D*: Fresh (*C*) and lyophilized (*D*) ribosomes extracted in 1.0% SDS by being stirred for 1 hour at room temperature centrifuged at $800 \times g$ for 3 minutes, and supernatant applied to gradient. Gradient construction and centrifugation characteristics as in Materials and Methods.

equally to RM protein sensitization where L-protein does not, and RS and RM protein respond very strongly to RS protein sensitization. These results suggest, semiquantitatively, that the nucleolar residue protein is a better antigen, weight/weight, than RM protein. The simplest explanation for this would be that RS protein is simpler with respect to contained



TEXT-FIGURE 3.—Gradient profiles of nucleolar RNA.

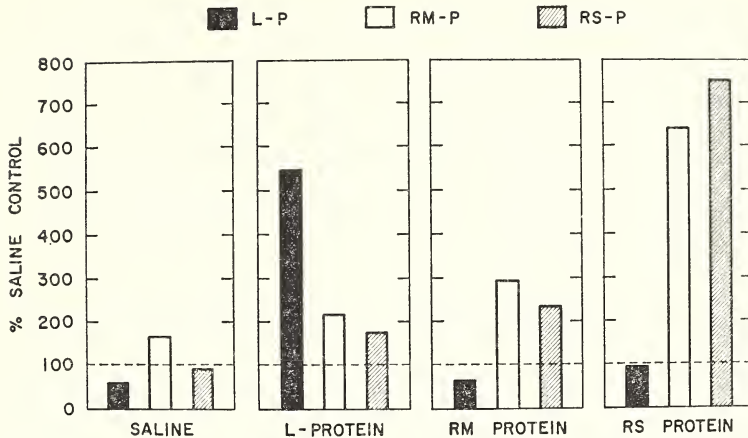
A, B, C: SDS extracted RNA profiles from lyophilized nucleolar preparations. Extraction procedures as in text-figure 2*D*.

D: *Solid line:* SDS extracted RNA from freshly prepared nucleoli. *Dashed line:* SDS extracted RNA from lyophilized ribosomes. Extraction and centrifugation procedures as in text-figure 2*D*.

components than RM protein, so that components which may be common would be present in higher concentration in the nucleolar residue proteins than ribosomal protein.

The results with skin testing were confirmed by interface precipitin experiments with an antiribosome sera (text-fig. 5). In this experiment, equivalence is obtained with about 100 μ g of RM protein/ml, while one-third that amount of residue protein gives the same result. Control experiments with nucleolar RNA and nucleolar L-protein show no response.

The nucleolar residue fraction apparently contains a higher concentration of available antibody than an equivalent amount of ribosomal protein.

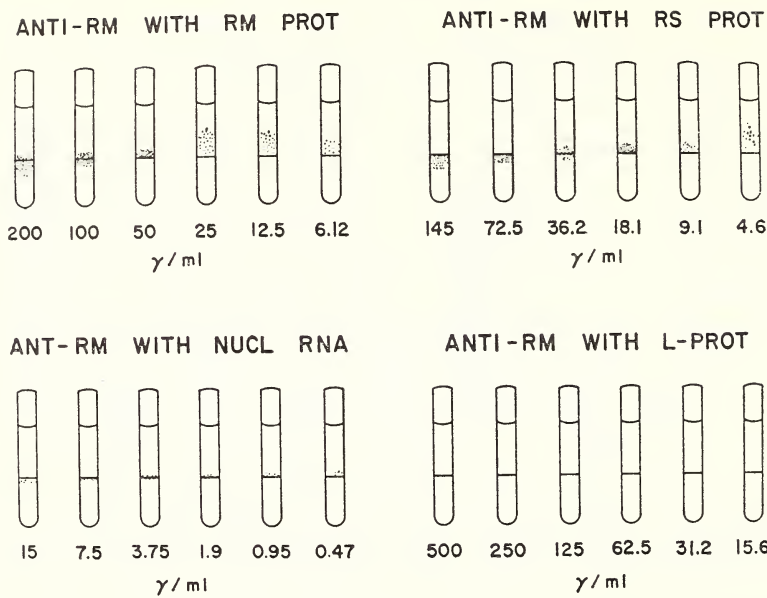


TEXT-FIGURE 4.—Skin test response to nucleolar and ribosomal antigens. Animals were sensitized with 50 μ g of antigen (ordinate) 7 days before injection of 10 μ g of test antigen in normal saline. The response of sensitized animals to test antigens is shown in the bars. Percent increase in skin thickness over that of the injection site of an equal volume of normal saline 24 hours after injection of test antigen. Dashed line represents the mean response to saline injection. The responses to saline as a sensitizing agent are not statistically significant. All other responses are significant at the 0.01 level or greater except for residue protein (RS-P) to L-protein (L-P) which is less than 0.1, and ribosomal protein (RM-P) to L-protein which is between 0.1 and 0.05 (t test).

Contamination of Nucleolar Preparations

Although the above results make the possibility unlikely, a series of experiments was carried out to test whether the nucleolar fraction could be contaminated with a significant amount of ribosomes, either adsorbed, or as microsomes. Oocytes were incubated with P^{32} for 5 hours and labeled ribosomes isolated. These labeled ribosomes were then added to the homogenate from which nucleoli were being isolated. Both nucleoli and ribosomes were isolated and the dilution of the added radioactivity by the unlabeled ribosomes measured, as well as the counts appearing in the nucleolar fraction. The results of this experiment and the calculated contamination of the nucleoli by adsorbed ribosomes are given in tables 1 and 2.

This type of experiment will demonstrate adsorbed ribosomes but will not necessarily demonstrate contamination by larger cytoplasmic fragments such as rough endoplasmic reticulum, which, although sparse in oocytes, still exists. A series of 28 randomly selected electron micrographs of sections of pellets of isolated nucleoli were examined by means of the Chalkley (21) technique for the relative volumes of the various components contributing to the pellet. This technique relies on the frequency of "hits" of a point on a 2-dimensional field. Magnifications of 4,000–35,000 were



TEXT-FIGURE 5.—Interface precipitin reactions between ribosomal and nucleolar fractions. Proteins in the concentrations indicated were dissolved in normal saline and layered over undiluted antiribosome serum. The interface is indicated by a *straight horizontal line* in each figurative test tube. The *dotted areas* represent the position and density of the precipitate formed.

Antiribosome serum was prepared from a rabbit after inoculation with 50 mg purified ribosomes in saline and Freund's adjuvant. After 3 weeks, a challenging dose of 1 mg of ribosomes was administered. The animal was exsanguinated and serum collected 1 week later.

TABLE 1.—P³²-labeled RNA appearing in isolated nucleoli and ribosomes

	cpm/ml	μg RNA/ml	Specific activity cpm/μg RNA
Nucleoli.....	11. 5	23. 7	0. 48
Ribosomes.....	1120. 0	24. 4	42. 4

TABLE 2.—Calculated contamination of nucleoli by RNA from adsorbed ribosomes or ribosomal derivatives

Basis of calculation*	Percent contamination
RNA weight.....	1. 14
Particle weight.....	0. 11
Protein weight.....	0. 38

*For purposes of calculation, the nucleoli and ribosomes were assumed to have the following RNA and protein composition: Nucleoli: 5 percent RNA, 95 percent protein; ribosomes: 50 percent RNA, 50 percent protein.

TABLE 3.—Microsomal contamination of isolated nucleolar pellets

Fraction	Number of hits	Percent of total
Nucleoli.....	13, 685	96. 19
Microsomes.....	35	0. 25
Yolk platelets.....	16	0. 11
Miscellaneous*.....	491	3. 45
Totals.....	14, 227	100. 00

*Most contaminants in this category were "jelly coat" membranes.

used and objects identifiable as rough reticulum (microsomes) were scored as contaminants. The results are shown in table 3.

Even though the total volume of microsomal vesicle is much larger than the sum of its attached ribosomal particles, the value obtained (0.25%) is extremely low and compares favorably with the biochemical data, in which the contamination, as determined for particles, was only 0.11%.

These experiments demonstrate that contamination of the nucleolar pellets with "microsomes," ribosomes, or ribosomal RNA precursors or products is very low.

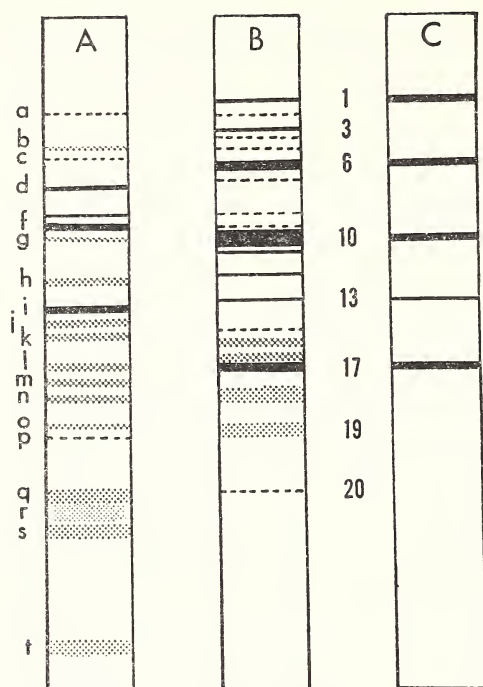
The biochemical experiments also demonstrate that ribosomal RNA synthesis and ribosome assembly are being carried out in this system. These experiments, when considered with previous studies using biochemical procedures, on RNA metabolism in starfish oocytes (8-10) and with those of Ficq (22) using autoradiographic techniques, provide a significant demonstration of the continuing ribosome synthesis in these oocytes and RNA metabolism in the nucleoli.

Disc Electrophoresis Comparisons of Ribosomes and Nucleoli

We compared ribosomal and nucleolar proteins by mixing different amounts of protein from one source with a constant amount from the other source. Six of these mixed samples with increasing amounts of ribosomal protein were run simultaneously. Comparing bands which showed a variation in intensity made it possible to locate the relative positions of the two kinds of proteins.

In all, the results reported below are based on data from more than 60 different samples. A composite diagram of the findings is shown in text-figure 6.

Pattern distribution for nucleoli is shown in text-figure 6B where up to 20 bands are found in intact nucleolar extracts. These bands are generally more sharply defined than those found for ribosomal proteins. Twelve of the bands were found on all 12 preparations of whole nucleoli run; 7 additional bands (2, 4, 5, 7, 8, 9, 20) were detectable among the various preparations.



TEXT-FIGURE 6.—Gel-electrophoretic patterns of ribosomal and nucleolar proteins.

A: Schematic distribution of bands compiled from 12 different experiments on extracts of ribosomal proteins. The relative sharpness and intensity of the bands are indicated. Band designations *a-t* are for text reference.

B: Distribution of bands from 12 nucleolar protein extracts prepared as in A. Bands are numbered 1-20 for text reference.

C: Band distribution found in residue protein fraction of the nucleolus. Only bands 1, 6, 10, 13, and 17 of the total nucleolar protein are found. Only band 17 appears to migrate coincidentally with ribosomal proteins.

The results from mixed samples of ribosomal and nucleolar proteins showed that band 17 coincided with *l*. The strong band N10 was located just below band *f*, but could coincide with the weak *g* band. It is most likely, but not positively shown, that N6 is below *b* and *c*. The mixed samples did not permit exact location of weaker nucleolar bands with respect to the ribosomal bands. Their relative position shown on the figure is based on measurements and calculation with respect to movement of the marker dye. It cannot be excluded that some of the weaker, sharply defined nucleolar bands coincide with the stronger, but more diffuse, ribosomal bands.

Among the basic proteins of the nucleolus are bands not occurring in the ribosomes. These include 1 and 3, 15 and 16.

The proteins extractable from ribosomes of different species (23, 24) give (10-30) bands by either starch gel or disc electrophoresis. According to Waller (23), each band is presumably a different protein. In agreement

with these findings the ribosomal protein from the starfish gives 16–20 bands on an acidic gel.

The pattern distribution shown in text-figure 6A is a composite of the bands found in 12 different experiments on ribosomal extracts. The relative intensity and sharpness of the bands are indicated. The pattern is essentially the same for acetic acid and LiCl extracts, except that band *g* is more intense and band *l* is closer to band *k* in the acetic acid extracts. In some gels, 4 weak bands (*a*, *c*, *p*, *r*) are more obvious than others, and bands *c*, *d*, *g*, and *p* appear as doublets.

The question arises of how much ribosomal protein can be detected in the nucleolar gels if the latter contains other basic proteins.

We have determined that, of the total nucleolar protein applied to the gel in one experiment, 50% disappeared from the sample region, either into the band pattern or into the buffer. In the ribosomal proteins, where there appeared to be considerable variation in the stainable material remaining in the sample gel region, the band patterns were such as to suggest that the material migrating was a random sample of the applied proteins. Until more precise data are available, we shall assume that similar protein will migrate in the same manner whether found in ribosomes or nucleoli. We have been able to show that 20 μ g of applied ribosomal protein is the minimal amount which will give detectable band patterns. As the normal amount of protein applied is 150–200 μ g, apparently no less than 10% of the applied protein could be ribosomal and still be detected. Therefore, in one experiment the gel was loaded with 750 μ g of nucleolar protein. The band pattern was somewhat distorted, and below N17 a few new or previously very weak bands were visible. No new bands could be located above the N17 region. There was no evidence for a band pattern corresponding to the triplet *l*, *m*, *n*. These might be covered in part by the intense bands in the 17 region, however.

Examination of the nucleolar subfractions did not reveal the presence of bands corresponding to ribosomal proteins in the nucleolus. The 1 M NaCl soluble fraction, which constitutes about 85% of the nucleolus, gave only a few faint bands when applied in amounts up to 200 μ g. The residue component, shown to have serological similarities to ribosomal protein, gave up to 5 bands which appeared to correspond to N1, 3, 6, 10 and 17, as shown in text-figure 6C.

On basic gels the ribosomal proteins gave no bands, whereas the nucleolar proteins gave only 1, presumably L-protein.

Our observations do not support the concept that the nucleolus of the starfish contains a complete spectrum of ribosomal protein. Some proteins may be common. Although bands N10 and N17 appear to be in the same position as *g* and *l*, these are not related in other than their abilities to migrate in acrylamide gels. If they were shown to be the same, it must then be concluded that the relative amount of these proteins in the nucleolus is different from the relative amount in the ribosomes.

Even if they are not the same, the basic proteins of the nucleolus are roughly similar to the ribosomal proteins in terms of number of bands and their mobility in acrylamide gels. This suggests that they are of approximately the same size and have about the same charge.

DISCUSSION

The RNA profiles for isolated starfish nucleoli are not unlike those reported for other forms (this volume) in that there is a large proportion of high molecular weight molecules (28S or larger) with a questionable contribution of the 18S category. The previous demonstrations that these nucleoli contain RNA of ribosome-like composition suggest that this large component is a ribosomal or ribosomal precursor RNA. We have already shown that these nucleoli contain an RNA fraction with the chemical and metabolic characteristics of transfer RNA (9, 10). This is consistent with the reports of Sirlin and colleagues that tRNA is present and that tRNA methylation takes place in nucleoli (25, 26).

The protein constituents of nucleoli are found to be very complex when examined by immunological and electrophoretic procedures. About 15% of the proteins of starfish nucleoli is basic; the rest are neutral or slightly acidic. On comparison of ribosomal with nucleolar proteins, we have found that, although there are some similarities, the nucleolus consists predominantly of proteins unlike those found in the ribosomes.

When we examined the literature on the RNA, DNA, and protein composition of nucleoli from various forms, we found that all nucleoli contained large quantities of protein. These data are summarized in table 4.

To compare more accurately the values from nucleolar preparations that contain differing amounts of DNA, the proportions of RNA and protein were recalculated after the DNA and equivalent amount of histone were subtracted. These values are shown in table 5.

TABLE 4.—Nucleic acid and protein content of nucleoli*

Source of nucleoli	RNA	DNA	Protein	Reference
Starfish oocyte	2. 1-4. 6	None	94	6
	3. 9	None	96	27
	5. 0	None	(95) †	7
	4. 4-15. 5	— ‡	(90)	28
Spider oocyte	7. 9	— ‡	(92)	29
Frog oocyte	8-17	— ‡	74-90	30
Pea seedlings	10. 8	6. 9	83	31, 32
Guinea pig liver	4. 0	9. 5	86. 5	33
Rat liver	2-4	12-18	(82)	34, 35
	3. 0	3. 0	94	36
Walker tumor	8. 0	7. 0	86	36

*All values % dry weight.

†Amount calculated by difference is shown in parentheses.

‡Value not determined.

TABLE 5.—RNA and protein content of nucleoli free of DNA*

Source of nucleoli	RNA	Protein	Reference
Starfish oocyte.....	5.0 10.0	95 90	7, 27 28
Spider oocyte.....	8.9	91	29
Frog oocyte.....	13.0	82	30
Pea seedling.....	13.0	87	31, 32
Guinea pig liver.....	5.7	94	33
Rat liver.....	4.0 3.0	96 97	34, 35 36
Walker tumor.....	9.0	91	36
Mean.....	8.0	92	

*Data taken from table 4. Mean values are used where ranges were given. All values as %.

The data from 9 different preparations show values of 3–13% RNA (mean = 8%) and 82–97% protein (mean = 92%). These values are similar to those found for whole nuclei (32, 37, 38). In 3 experiments in which basic versus nonbasic proteins were determined, nonbasic (and therefore nonribosomal) proteins make up at least 50% of nucleolar protein (table 6).

Other nucleolar constituents include a variety of enzymes, summarized by Vincent, Siebert *et al.*, and Tsukada and Lieberman (27, 39, 40, and this volume). Such determinations suffer from lack of purity of nucleolar preparations so that it is difficult to correlate the exact spectrum of enzymes in a way which would illuminate the problems of nucleolar function. At this time their presence can only be noted.

Thus the evidence provided by the starfish material reported above is consistent with data from other sources that the nucleolus contains a large population of 28S RNA (ribosomal) molecules and tRNA. With respect to the presence of other ribosomal components, however, the data on proteins are less satisfactory.

No matter what source, most of the nucleolus is protein (more than 90%). Analyses of this protein have shown that it is at least 50%, and usually more, of the nonbasic type. Even the nucleolar basic proteins, as shown in the starfish material, may differ markedly from the ribosomal basic proteins. No evidence is currently available as to the role of these other protein constituents of the nucleolus. Their consistent presence in large amounts suggests a nucleolar function not yet understood.

TABLE 6.—Amounts of basic and nonbasic protein in nucleoli*

Source of nucleoli	Basic	Nonbasic	Reference
Starfish oocyte.....	15	85	10
Pea seedlings.....	28	72	31
Guinea pig liver.....	50	50	33

*All values as %. Values for basic protein were corrected by subtraction of an equivalent weight % of DNA, when present, to account for DNA-associated histone.

The data given here do not support the concept that the nucleolus of the oocyte is the site of significant storage of intact ribosomes, the site of storage of 18S ribosomal RNA, or of the complete spectrum of ribosomal proteins. The antigenic similarities, confirmed by electrophoretic analysis, do suggest the presence of some common proteins. The nucleolar role in ribosome biogenesis in this material would seem to be limited to 28S ribosomal RNA storage plus the provision of certain proteins. This might be in the form of a precursor RNP particle such as the chloramphenicol particle found in bacterial systems (41).

RESUMEN

Los análisis de proteína y ácido nucleico del material de la estrella de mar *Asterias forbesii* no apoyan el concepto de que el nucleolo del ovocito sea el lugar de almacenamiento significativo de ribosomas intactos o el lugar de almacenamiento del RNA ribosómico 18S o el lugar de almacenamiento del espectro de las proteínas ribosómicas. Las semejanzas antigénicas entre las proteínas ribosómicas y las nucleolares, confirmadas mediante electroforesis en gel, sugieren que existen algunas proteínas comunes. El papel nucleolar en la biogénesis del ribosoma en este material parece que está limitado al ARN 28S, más la provisión de ciertas proteínas. Esto podría ser en forma de una partícula RNP del precursor ribosómico similar a la partícula de cloramfenicol que se ha encontrado en sistemas bacterianos.

REFERENCES

- (1) VINCENT, W. S.: Structure and chemistry of nucleoli. *Int Rev Cytol* 4: 269-298, 1955.
- (2) SWIFT, H.: Studies on nucleolar function. *In* A Symposium on Molecular Biology (Zirkle, R. E., ed.). Chicago, Univ Chicago Press, 1958, pp 266-303.
- (3) SIRLIN, J.: The nucleolus. *Progr Biophys* 12: 25-66, 1962.
- (4) PETERMANN, M. L.: The Physical and Chemical Properties of Ribosomes. New York, Elsevier, 1965.
- (5) BROWN, D. D.: The nucleolus and synthesis of ribosomal RNA during oogenesis and embryogenesis of *Xenopus laevis*. *Nat Cancer Inst Monogr* 23: 297-309, 1966.
- (6) VINCENT, W. S.: The isolation and chemical properties of the nucleoli of starfish oocytes. *Proc Nat Acad Sci USA* 38: 139-145, 1952.
- (7) BALTUS, E.: Observations sur le rôle biochimique du nucléole. *Biochim Biophys Acta* 15: 263-267, 1954.
- (8) VINCENT, W. S.: Heterogeneity of nuclear ribonucleic acid. *Science* 126: 306-307, 1957.
- (9) VINCENT, W. S., and BALTUS, E.: The ribonucleic acids of nucleoli. *In* The Cell Nucleus (Mitchell, J. S., ed.). London, Butterworth & Co., 1960, pp 18-23.
- (10) VINCENT, W. S.: The nucleolus. *In* Genetics Today. *Proc XI Int Cong Genetics* (Geerts, S. J., ed.). London, Pergamon Press, 1964, vol 2, pp 343-358.
- (11) ———: A biochemical approach to cell morphology. *In* New Approaches in Cell Biology (Walker, P. M. B., ed.). New York, Academic Press Inc., 1960, pp 89-94.
- (12) BARBU, E., and PANIJEL, J.: Concentration et propriétés des anticorps anti-acide ribonucleique. *C R Acad Sci (Paris)* 252: 3157-3159, 1961.

- (13) PANIJEL, J.: Précipitation des ribopolynucléotides homopolymères par les divers anticorps du sérum antiRNA. C R Acad Sci (Paris) 257: 3064-3067, 1963.
- (14) BIGLEY, N. J., DODD, M. C., and GEYER, V. B.: The immunologic specificity of antibodies to liver ribosomes and nuclei. J Immun 90: 416-423, 1963.
- (15) TRAKATELLIS, A. C., STINEBRING, W. R., and AXELROD, A. E.: Studies on delayed hypersensitivity to proteins and protein conjugates. J Immun 91: 591-596, 1963.
- (16) REISFELD, R. A., LEWIS, U. F., and WILLIAMS, D. E.: Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature (London) 193: 281-283, 1962.
- (17) LEBOW, P. S., COX, E. C., and FLAKS, J. G.: The chromosomal site specifying a ribosomal protein in *Escherichia coli*. Proc Nat Acad Sci USA 52: 1367-1374, 1964.
- (18) GALL, J. G.: Nuclear RNA of the salamander oocyte. Nat Cancer Inst Monogr 23: 475-488, 1966.
- (19) PENMAN, S., SMITH, I., HOLTZMAN, E., and GREENBERG, H.: RNA metabolism in the HeLa cell nucleus and nucleolus. Nat Cancer Inst Monogr 23: 489-512, 1966.
- (20) PERRY, R. P.: On ribosome biogenesis. Nat Cancer Inst Monogr 23: 527-545, 1966.
- (21) CHALKLEY, H. W.: Method for the quantitative morphologic analysis of tissues. J Nat Cancer Inst 4: 47-53, 1943.
- (22) FICQ, A.: Metabolism of the nucleolus during oogenesis, maturation, and early steps of embryonic development in echinoderms. Nat Cancer Inst Monogr 23: 311-323, 1966.
- (23) WALLER, J. P.: Fractionation of the ribosomal protein from *Escherichia coli*. J Molec Biol 10: 319-336, 1964.
- (24) WORK, T. S.: Electrophoretic separation of the proteins of ribosome subunits and of encephalomyocarditis virus. J Molec Biol 10: 544-545, 1964.
- (25) SIRLIN, J. L., JACOB, J., and TANDLER, C. J.: Transfer of the methyl group of methionine to nucleolar ribonucleic acid. Biochem J 89: 447-452, 1963.
- (26) SIRLIN, J. L., JACOB, J., and BIRNSTIEL, M. L.: Synthesis of transfer RNA in the nucleolus of *Smittia*. Nat Cancer Inst Monogr 23: 255-270, 1966.
- (27) VINCENT, W. S.: Some studies on differentiation and development of the oocyte. In The Beginnings of Embryonic Development (Tyler, A., von Borstel, R. C., and Metz, C. B., eds.). Washington, D.C., AAAS, 1957, pp 1-22.
- (28) EDSTRÖM, J.-E., GRAMPP, W., and SCHÖR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. J Biophys Biochem Cytol 11: 549-557, 1961.
- (29) EDSTRÖM, J.-E.: Composition of ribonucleic acid from various parts of spider oocytes. J Biophys Biochem Cytol 8: 47-51, 1960.
- (30) FINAMORE, F. J.: The nucleic acids—metabolic key to development? Quart Rev Biol 36: 117-122, 1961.
- (31) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and FLAMM, W. G.: On the chemistry and organization of nucleolar proteins. Biochem Biophys Acta 87: 111-122, 1964.
- (32) BIRNSTIEL, M. L., and HYDE, B. B.: Protein synthesis by isolated pea nucleoli. J Cell Biol 18: 41-50, 1963.
- (33) MAGGIO, R., SIEKEVITZ, P., and PALADE, G. E.: Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic sub-fractions. J Cell Biol 18: 293-312, 1963.
- (34) LITT, M., MONTY, K. J., and DOUNCE, A. L.: Isolation and properties of rat liver cell nucleoli. Cancer Res 12: 279, 1952.
- (35) DOUNCE, A. L.: The isolation and composition of cell nuclei and nucleoli. In The Nucleic Acids (Chargaff, E., and Davidson, J. N., eds.). New York, Academic Press Inc., vol II, 1955, pp 93-153.

- (36) BUSCH, H., DESJARDINS, R., GROGAN, D., HIGASHI, K., JACOB, S. T. MURAMATSU, M., RO, T. S., and STEELE, W. J.: Composition of nucleoli isolated from mammalian cells. *Nat Cancer Inst Monogr* 23: 000-000, 1966.
- (37) MONTY, K. J., LITT, M., KAY, E. R. M., and DOUNCE, A. L.: Isolation and properties of liver cell nucleoli. *J Biophys Biochem Cytol* 2: 127-145, 1956.
- (38) MAGGIO, R., SIEKEVITZ, P., and PALADE, G. E.: Studies on isolated nuclei. I. Isolation and chemical characterization of a nuclear fraction from guinea pig liver. *J Cell Biol* 18: 267-291, 1963.
- (39) SIEBERT, G., VILLALOBOS, J. G., RO, T. S., STEELE, W. J., LINDENMEYER, G., ADAMS, H., and BUSCH, H.: Enzymatic studies on isolated nucleoli of rat liver. *J Biol Chem* 241: 71-78, 1966.
- (40) TSUKADA, K., and LIEBERMAN, I.: Synthesis of ribonucleic acid by liver nuclear and nucleolar preparations after partial hepatectomy. *J Biol Chem* 239: 2952-2956, 1964.
- (41) KURLAND, C. G., NOMURA, M., and WATSON, J. D.: The physical properties of the chloromycetin particles. *J Molec Biol* 4: 388-394, 1962.

DISCUSSION

Busch: We have carried out studies on the amino acid composition and amino terminals of proteins of the nuclear residue fraction, which contains the nucleolus and the fine filaments appearing to migrate to the nuclear membrane (Busch, *Histones and Other Nuclear Proteins*, Academic Press, 1965). We did not find significant differences between either the 5 amino terminals, or the amino acid composition of these proteins and the ribosomal proteins. With respect to ribonuclease, it was necessary to add PVS to the initial medium for isolation of the nuclei in order to obtain undegraded RNA from our nucleoli. With your conditions for extraction of the nuclei, *i.e.*, with 2 M NaCl and dilution to 0.10 M NaCl, any deoxyribonucleoprotein would necessarily precipitate. If this is so, it wouldn't be particularly surprising to have a high lysine content; this would suggest that your protein fraction is a mixture of histones and acidic proteins.

Vincent: The contribution of DNA histones to the basic protein fraction we have analyzed is negligible. This follows from the fact that the total DNA content of a starfish oocyte nucleus is less than 1% of the amount of RNA found in the nucleolus, and one would assume that the histones would be no more than the DNA in actual amount. As the L-protein fraction is 85% of nucleolar dry weight, DNA-associated proteins would therefore not contribute an appreciable amount. Baltus has shown that these nucleoli contain considerable amounts of ribonuclease, predominantly in the inactive state (*Biochim Biophys Acta* 55: 82-91, 1962). The sedimentation profiles we have shown do not indicate appreciable degradation of high molecular weight RNA when SDS denaturation of protein was used. Complete absence of ribonuclease effect cannot be ruled out, of course.

von Borstel: Was any of the data you obtained on ribosomes and proteins from mature oocytes also obtained from actively growing oocytes? Do you have similar data for young oocytes?

Vincent: In *Asterias forbesii* the oocytes start proliferating in early November and thereafter grow rapidly until around April. From then on, growth is slower until complete maturity is reached. We have studied oocytes taken from December and January starfish and have found no difference from the results presented here with respect to serological reactions or RNA synthesis, except for rate. We know that nucleoli taken from oocytes during the April-July period are still carrying out synthesis of RNA, and that newly labeled RNA appears in intact cytoplasmic ribosomes. We conclude that these nucleoli are still functioning in RNA synthesis as they were in younger oocytes, although perhaps at a slower rate.

Birnstiel: Some time ago we reported that the amino acid composition of the nucleolar residual protein resembled closely that of ribosomal proteins (Birnstiel, Chipchase, and Flam, *Biochim Biophys Acta* 87: 111-122, 1964). Dr. Busch has confirmed this finding and extended it to the end-terminal amino acids which are also indistinguishable from those of ribosomal proteins. It is interesting that in your experiments the residual nucleolar fractions also show some immunological cross-reaction with ribosomal proteins. These nucleolar proteins are characteristically residual, that is, they are not readily solubilized. I wonder whether the failure to identify similar bands in the electrophoretic analysis is a consequence of the proteins being not solubilized.

Vincent: We have seriously studied the problem as to whether migration of protein from the sample gel into the higher cross-linked fraction could be a selective process which would prevent us from demonstrating the ribosomal bands if they were present in the nucleolus. The best control we could conceive of was the mixing of relatively small quantities of ribosomal protein into the solubilized nucleolar protein, and then seeing if the ribosome bands could be detected. Ribosomal bands could be detected if approximately 10% ribosomal protein was added. The major ribosome bands were detectable when only 20 μ g of protein was applied to the gel. Except for the band pointed out above, we could not detect characteristic ribosomal bands even when 750 μ g of nucleolar protein was applied. Therefore, we believe we are justified in concluding that, although the nucleolus contains proteins of similar size and/or charge characteristics of those of ribosomes, these proteins are different.

We have also measured the proportion of added protein which leaves the sample gel. This is variable, as can be seen in the photographs we have shown. Our analyses have revealed that usually 50% or more of the applied protein will migrate. Some of this may move into the buffer, of course. But we have no evidence that migration from the sample gel is selective with respect to the population of basic proteins applied.

We have also examined in detail the problem of solubilizing the proteins in both ribosomes and nucleoli. In the 3 different extracting procedures used, only one difference in the resulting bands could be detected in proteins from each structure. These differences took the form of bands which aggregated in 1 extracting medium, and not in others, thus giving a change in the appearance of 1 or 2 bands as the case might be. The characteristic band patterns of nucleoli and ribosomes were always readily distinguishable.

Perry: I would like to bring up again the point that Dr. von Borstel raised about the rapidly growing versus the mature oocyte and its bearing on the data on base composition you presented. Edstrom (*Biochem Biophys Res Commun* 18: 595-599, 1965) showed that there is a shift in the base composition of nucleolar RNA between the rapidly growing and the mature oocyte, and that the similarity between rRNA and nucleolar RNA, which you fail to demonstrate in mature oocytes, is quite evident in the immature, rapidly growing oocyte. Therefore, it is important to know, in your experiments on the characterization of protein, whether because you use mature oocytes, in which Edstrom has shown the proportion of ribosome-like material to be much less, you find such a dissimilarity between nucleolar and ribosomal proteins. This is an extremely important point.

Vincent: I agree that it is important to consider differences which may exist between young and older oocytes, and I am sorry that Dr. Edstrom could not attend this meeting to contribute to this question. First, we do demonstrate a fraction of the RNA of the nucleolus which has ribosome-like base composition. This was demonstrated on both stored nucleolar RNA and on newly synthesized RNA in oocytes which may be called "mature." We also show a significant amount of the total nucleolar RNA lying in the 28-30S region. Our analyses have a certain advantage over Dr. Edstrom's in that we are able to fractionate the RNA components of the nucleolus and show differences in composition of these fractions both in stored and

newly synthesized fractions. I pointed out as early as 1957 (27) that the nucleolus contained different fractions of RNA, and that these fractions varied during the growth of the oocyte. Baltus and I showed in 1960 (9) that one of these fractions was soluble RNA. Dr. Edstrom's analyses essentially confirm our early observations.

Penman: Dr. Vincent, I'm a little concerned about your method of RNA extraction from the nucleolus. What is the absorbing material seen at the bottom of the gradient you showed?

Vincent: We place a cushion of 2 ml of 2 M sucrose at the bottom of our gradients and then record the optical density of the gradient by pumping heavy sucrose into the bottom of the centrifuge tube. The sharp rise at the bottom of the tracing represents Schlieren scattering at the interface between the 2 M cushion and the 1.4 M sucrose at the bottom of the gradient.

Penman: The reason I mentioned this is that SDS alone as a protein-denaturing agent has to be used cautiously, since sometimes the denaturation may not be complete. For instance, I believe that SDS does not break up much of DNA-histone complexes. Actually some of my own experiments suggest that the earliest RNA in the nucleolus may not be broken free of protein in the presence of SDS. Perhaps one should not assume that, because SDS releases all RNA from protein in mature ribosomes, the linkages of RNA to protein in the nucleolus would be the same and that complete extraction can be achieved with SDS alone. I believe there may be something qualitatively different about the organization of the earliest RNA in the nucleolus. I would like to know if you have checked the recovery just to establish that in your system you are getting back all the RNA in the nucleolus.

Vincent: I don't believe we checked the absorption spectrum of the tubes which would have been collected at the interface zone in the experiments shown. When we have checked it with ribosomes, our recovery is certainly better than 90%. With the nucleoli, we have estimated that recovery is 70-80%.

Penman: That is the cautionary point I would like to make. The 20% of the RNA lost might be quite special. In other words, the losses in SDS treatment can easily be selective, and the fact that the RNA from ribosomes is recovered better than that from nucleoli would be a source of worry. Also, I'm always concerned when people talk about ribosomal proteins, because under some conditions apparently ribosomes will pick up nonspecific proteins from the cytoplasm. Would you care to comment about this?

Vincent: This is also one of the problems we are worried about, and so we have carried out extensive washing procedures. We followed the procedure developed by Dr. Bock of the University of Wisconsin. He has shown that several washings after isolation of ribosomes in presence of 0.3-0.5 M ammonium chloride provides very clean ribosomes by removing nascent and absorbed proteins. We made a series of gel fractionations on the ribosomal proteins in which we washed or did not wash them with ammonium chloride to see whether we could detect any differences in ribosomal protein spectra. Actually, in the ribosomes of the starfish, we did not detect any differences, whether we washed them or not. I can add that in the work we are doing on yeast we can show detectable differences, depending on whether we wash the ribosomes in high salt solution.

Schultz: I am concerned about the proportion of the total ribosomes which will be formed by an oocyte that still remains to be synthesized at the time you are measuring your nucleoli. It seems to me that in considerations of nucleolar function, one must think in terms of kinetics, and your analogy with the chloroamphenicol bacteria may be a very good one for this system, which then would differ from other nucleoli.

Vincent: I cannot answer the question with respect to the proportion of ribosomes yet to be formed in the oocyte at the time we are doing our experiments. We have tested nucleoli from young oocytes by serological means at periods when ribosome synthesis, as measured by incorporation of labeled RNA precursor, is proceeding at a more rapid rate than in the cells used for the data presented here. The nucleolar proteins extractable by the same technique as ribosomal proteins gave the same rela-

tive reactivity as those described above. In other words, the proportion of common antigen between the nucleolar residue fraction and ribosomal protein did not appear to increase at this stage.

Schultz: The question I ask is simpler. A total amount of ribosomes is going to be made in the life of an oocyte. Most of these ribosomes presumably have already been synthesized by the time you make your measurement. You are probably working with the nucleolus at the time any feedback mechanism would be in control of function.

Vincent: Let me make clear that in our experiments where we have attempted to compare ribosomal and nucleolar proteins with serological techniques as well as on acrylamide gels, we have dealt with subfractions of the nucleolus, *i.e.*, that fraction of the nucleolus soluble under the same conditions solubilizing a minimum of 90% of the ribosomal protein. As there is a larger portion of the nucleolus not solubilized under these conditions, or if so, never migrating on the acid gel, the procedure acts to concentrate proteins found in the nucleolus which are most like ribosomal protein (100–200 μ g protein from 50–100 mg of nucleoli). This is the fraction of nucleolar protein being compared to total ribosomal protein. When we show that only a fraction of this already small proportion of nucleolar protein may have common antigenic or migratory properties with ribosomes, I believe we are justified in concluding that we cannot demonstrate the spectrum of ribosomal proteins in the nucleolus.

Busch: One of the things Dr. Siebert will probably indicate later is that the nucleolus possesses a number of enzymes which may move in your gel patterns, and these enzymes are not present in ribosomes. This could account for one of your discrepancies.

Brown: There is no need to belabor the problem that these oocyte nucleoli are not typical nucleoli. Furthermore, the population of oocytes might be mixed and contain immature ones still synthesizing RNA along with mature ones no longer synthetically active. The chemistry is done on mature ones, which represent the bulk of the preparation. From your data, I'm puzzled to see how you draw your conclusions. The RNA isolated from your nucleoli is high molecular weight RNA which appears to sediment as the rRNA precursor. Your gradients resemble those of Dr. Busch and others. The evidence for your conclusion that the nucleolus is not the site of rRNA synthesis is that you don't find any 18S RNA in your preparations. This is also a common finding and not incompatible with the nucleolus as the site of its synthesis. Possible mechanisms for this paradox will be discussed later in the Symposium. The other question I have deals with your protein bands. In the B and C gels, it looked as though the nucleolus has extra bands, but also many in common with ribosomal protein. In comparison of the two, it seemed as though over half the bands in the ribosomal protein were present in the nucleolus. Will you comment, particularly on the difference between the B and C gel patterns?

Vincent: I hope we will find a better way of reproducing the band pattern from acrylamide gels, so that we can make the differences more evident. But we are fully convinced that the single band shown earlier is the only one of more than 25 major bands found in the ribosomal and nucleolar basic proteins which migrates coincidentally on the gels. That some of the minor bands may be coincident is not completely ruled out. We are further impressed by the agreement between such diverse techniques as gel electrophoresis and two different serological procedures.

We have clearly stated that one of the RNA fractions of these nucleoli resembles rRNA in both its base composition and its velocity sedimentation characteristics. What we have not been able to demonstrate is ribosomal precursor particles or ribosomal proteins in nucleoli.

Synthesis of Transfer RNA in the Nucleolus of *Smittia*^{1, 2}

J. L. SIRLIN, J. JACOB, and M. L. BIRNSTIEL, *Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland*

SUMMARY

In larval salivary cells of a chironomid and 4S ribonucleic acids.—*Nat Cancer the nucleolar synthesis, isolated within Inst Monogr 23: 255–270, 1966.* the cell by chemical means, is of >28S

FOLLOWING THE progressive elucidation of the role of different RNAs in the last few years, efforts were directed to locate aspects of the formation of the RNAs in the various cell structures. Biochemistry of many elegant genetic systems indicates certain formative pathways of the molecules, mainly at the chemical level. Chemical work provides information assigning these pathways to different structures in the cell. Curiously enough, the nucleolus has been the last major organelle involved in RNA synthesis to be explained, though its earliest documentation in 1781 makes it historically one of the first to be recognized. The nucleolus is ascribed with the synthesis of precursors of ribosomal RNA (1). Our contribution is that the nucleolus also synthesizes 4S RNA (2–4).

EXPERIMENTAL

Biological material.—The conditions of breeding of the chironomid *Smittia parthenogenetica* used in the experiments have been described (3). Conventional stage I occurs during the third larval instar. The second (wing) body segment has the same length and pigmentation as the third segment. Stage III, which is the one used except when the previous stage is mentioned, occurs in the fourth (last) instar just before metamorphosis.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This work was supported by the Damon Runyon Memorial Fund for Cancer Research (DRG-579), Agricultural and Medical Research Councils, and British Empire Cancer Campaign for Research.

The enlarged second segment is twice the length of the contracting third segment, the pigment becoming sparser in the second segment and more condensed in the third. Stage III is more advanced than in the original description (5) and therefore closer to stage IV with maximum nuclear and nucleolar size. Recalculation of the nucleolar volume more fittingly as for an oblate spheroid, rather than prolate spheroid, shows that by stage III the nucleolus has grown more since earlier stages than as previously (5) calculated.

Previous descriptions of the nucleus of *Smittia* include the cytology (6), ultrastructure (7), and autoradiography observations on the course of synthesis of DNA, RNA, and protein at the microscopic (2, 5, 6) and submicroscopic (8) levels.

Incubation of glands.—The technique of mass incubation of intact glands in synthetic medium was described (3, 4). Glands incubated in this medium and finally in the presence of tracers served as controls; the inhibitors added in the experimental series are detailed in the text-figures and tables.

The criteria of viable function include the high rate of DNA, RNA, and protein syntheses which are qualitatively similar to those *in vivo* (6), the secretion of ^{35}S -labeled mucoprotein (9) and vital staining (3).

Each experiment consisted of a series of incubations covering the interval of maximum diurnal metabolic activity. In quantitative experiments, the control and experimental series were alternated during this interval on the same day. Effects at the molecular level of rejuvenation after explantation (10) seemed irrelevant, at least for the nucleolus, since similar RNAs were synthesized in stages I and III (text-figs. 1 and 2) and differences after stage III are unlikely.

Chemicals.—Uridine-5- ^3H (21.5 and 22.4 c/mmole) and L-methionine (methyl- ^{14}C) (29.1 mc/mmole) were purchased from Radiochemical Center, Amersham, England.

5,6-Dichloro- and 4,5,6-trichloro-1-(β -D-ribofuranosyl)benzimidazole (DRB and TRB, respectively) and actinomycin D were gifts of Merck Sharp & Dohme, Rahway, New Jersey.

Chemical procedure.—At the end of radioactive incubation the glands were frozen and the RNA was extracted with cold phenol and 1% sodium dodecyl sulfate (SDS) followed by phenol at 60 C (4). Carrier *Xenopus* RNA, extracted by the same procedure, was used to coprecipitate the radioactive RNA. The yield of RNA was better than 80% in terms of radioactivity and did not materially improve after re-extraction with hot phenol and SDS. Particulars of the sucrose sedimentation gradients and determination of radioactivity have been given previously (4).

The incorporation of tracers into RNA represented total synthesis of the molecule. All four ribonucleosides were equally effectively incorporated, as shown by autoradiography and application of the ribonuclease test (4) which removed the bulk of cell radioactivity except for sporadic radioactivity in chromosomes. This sporadic radioactivity resembles that

after small doses of thymidine- ^3H (11) and is therefore probably in DNA. Sucrose gradient sedimentation patterns of radioactive RNA qualitatively similar are obtained with tracer uridine or guanosine (2). Paper chromatography analysis indicated that the newly synthesized 4S RNA from untreated glands contained 76% of uridine- ^{14}C incorporated as UMP; in total ^{14}C -RNA from benzimidazole-inhibited glands, consisting mainly of 4S RNA, the value was 89% (4). Counts due to terminal pCpCpA turnover can therefore be only minimal.

Transmethylation from methionine(methyl- ^{14}C) to RNA was established by chromatography as being predominant over direct incorporation of C_1^{14} -units (3). Cytochemically, the (nucleolar) radioactivity acquired from methionine was not affected by amino acid cleavage, while the bulk was ribonuclease-sensitive and puromycin-insensitive (3). Chemically, radioactivity in 4S RNA contributed by methionyl-RNA was estimated at <2%, and the amino acid was routinely cleaved at a high pH(4). Radioactivity due to protein contamination in this region of the gradient was about 5% after hydrolysis of the RNA (4). For autoradiography, puromycin was used as inhibitor of protein synthesis. It was shown that puromycin did not alter the sucrose gradient sedimentation pattern of RNA synthesized in the presence of uridine- ^{14}C or the pattern of RNA methylation presented in text-figure 3 (4).

The salivary glands were dissected free from extraneous tissue, except for minimal remnants of fat bodies that could not be practicably dissected but they had insignificant relative RNA label as determined by autoradiography. Bacterial contamination was not found by electron microscopy either in the incubation medium, which contained antibiotics, or attached to glands. No residual nuclease activity was observed after adding 28S ^{14}C -RNA (16 μg ; 1.6×10^5 counts/minute), prepared from *Xenopus* tadpoles and semipurified by the routine procedure, to 215 pairs of unlabeled glands, and the RNA was extracted. A single sharp sedimentation peak was obtained with 14,700 counts/minute at the apex.

The sedimentation values ($S_{20,w}$) given in the text-figures correspond to *Xenopus* carrier RNA, *i.e.*, 28S, 18S, and 4S. The actual values in *Smittia*, determined from larvae at stage III or older when the gut no longer contains food, were 30 and 19 for the ribosomal RNAs. The RNA was extracted with phenol-SDS, and high ionic strength (0.2 M NaCl) was maintained, as well as high temperature was avoided, throughout procedures to prevent loss of secondary structure. *Xenopus* RNA was used as internal control for the determination in the Spinco Model E centrifuge.

Autoradiography.—Four to eight glands in each batch prepared for chemical analysis were checked visually for incorporation with the use of autoradiography as described before (3).

To measure relative intracellular radioactivities (table 1), grains were counted on the class of cells showing high incorporation. That this class is representative of the total incorporation is clearly appreciated

when cell counts at *all* degrees of incorporation are arranged in a histogram (12). Cells with exceptionally high incorporation that showed radioactive RNA in the nuclear sap were avoided. This RNA derives from nucleoli and chromosomes, as can be seen after inhibition either of nucleolar or chromosomal synthesis with actinomycin or benzimidazole, respectively.

Isolation of radioactive nucleolar RNA.—Isolation is effected by selective inhibition of incorporation into chromosomal and cytoplasmic RNA by the use of DRB and TRB (13). The effects are shown in table 1 for a typical experiment (12).

TRB acts antagonistically to purine ribosides (14). DRB and TRB finally inhibit RNA synthesis completely but reversibly (13). Why the nucleolar synthesis was less inhibited (table 1) is not known. This is

TABLE 1.—Quantitative autoradiography of RNA synthesis

Glands were incubated first in synthetic medium for 110 minutes, and then in medium with uridine-³H (91 μ c/ml) for 45 minutes, either without (controls) or with DRB and TRB (40 μ g/ml each) present throughout.

Whereas the nucleolar incorporation during inhibition (65%) is into RNA, as described in Chemical Procedure, most of the residual chromosomal incorporation (11%) can be into DNA. Sucrose gradient sedimentation patterns of the RNA are given in text-figure 1.

	Control (*)	DRB-TRB (*)	Inhibition (%)
Nucleolus (†).....	360.3 \pm 39.5	232.4 \pm 34.7	35.5
Chromosomes (†).....	185.2 \pm 38.7	20.9 \pm 3.7	88.7
Cytoplasm (‡).....	(157.6 \pm 16.4)	(8.0 \pm 4.0)	94.9

* Net count \pm standard error of mean.
† Total grain count.
‡ Grain count over about one fourth of cytoplasm, multiplied by 4.

TABLE 2.—Effect of DRB and TRB on RNA polymerase

The control reaction mixture (10 minutes at 30 C) contained in 0.5 ml: 1.6 mM spermidine; 2.5 mM MnCl₂; 0.8 or 0.08 mM each of GTP, UTP, and CTP; 0.8 or 0.08 mM ATP-8-¹⁴C (1c/mole); 0.1 M Tris buffer, pH 7.5; 1.25 μ g mouse DNA; and RNA polymerase ex *Micrococcus lysodeikticus* [step VI in (16)].

	m μ moles ¹⁴ C-AMP incorporated into RNA*	
	Experiment 1	Experiment 2
Control (0.08 mM nucleotide)		0.823
“ (0.8 mM nucleotide)	2.261	
plus DRB (0.156 mM) (50 μ g/ml).....	2.106	0.785
“ TRB (50 μ g/ml).....	1.990	0.683
“ DRB (0.312 mM) (100 μ g/ml).....	1.887	
“ TRB (100 μ g/ml).....	2.164	
“ DRB+TRB (50 μ g/ml each).....	2.029	0.738

*In experiment 1 the DNA was transcribed twice into RNA, in experiment 2 to the extent of 80%.

not a peculiarity of the system since it has been observed also in starfish oocyte nucleoli [(15) p 159].

In an assay done by Dr. J. O. Bishop in our laboratory, no interference by DRB and TRB was found on the transcription of DNA by RNA polymerase. The results are summarized in table 2.

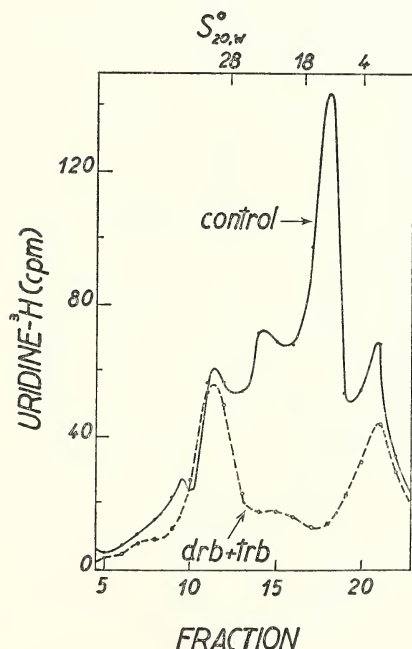
RESULTS

Species of RNA Synthesized in the Nucleolus

Sucrose gradient sedimentation patterns of RNA newly synthesized in the salivary gland show sRNA, ribosomal RNA, and RNA larger than 28S, apparently superimposed on a highly polydisperse RNA, with peaks varying in relative size in different experiments. A typical pattern is shown in text-figure 1. Table 1 indicates the respective contribution of nucleolus, chromosomes, and cytoplasm to the total RNA in the sedimentation.

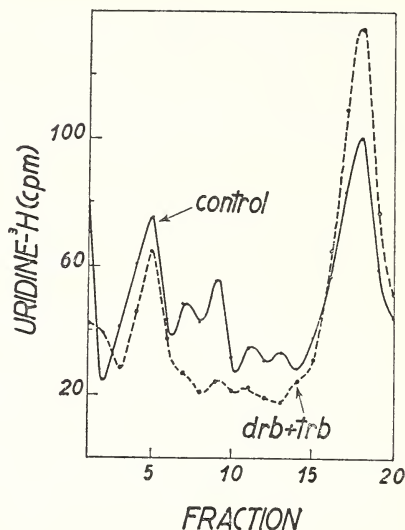
In glands in which the newly synthesized RNA is mainly nucleolar, as the result of treatment with DRB and TRB (table 1), the RNA consists of sRNA and RNA larger than 28S (text-fig. 1). The sRNA is a *de novo* synthesis (see Experimental).

In similar experiments carried out on growing larvae of stage I (text-fig. 2) the newly synthesized RNA contains sRNA and RNAs of large molecular weight as in grown larvae (text-fig. 1). The profile of RNA



TEXT-FIGURE 1.—Sucrose gradient sedimentation pattern of RNA radioactivity. Control glands were incubated first in synthetic medium for 110 minutes and then in the same medium with uridine- ^3H ($91\text{ }\mu\text{c/ml}$) for 45 minutes. Glands from another batch were similarly incubated in the presence of DRB and TRB ($40\text{ }\mu\text{g/ml}$ each). The counts for 199 pairs of treated glands are adjusted to 84 control pairs.

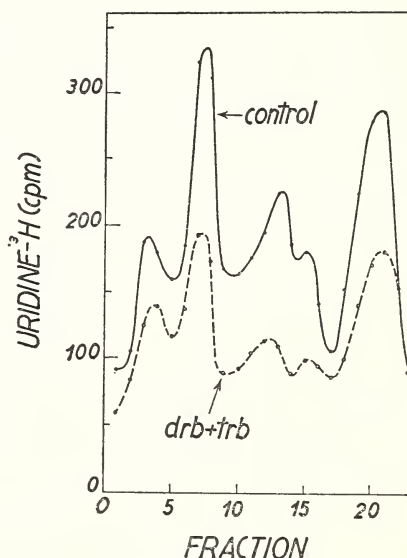
TEXT-FIGURE 2.—Sedimentation pattern of RNA in young larvae. Glands from stage I larvae were incubated as described in text-figure 1. The counts for 266 pairs of treated glands were adjusted to 219 pairs in controls.



synthesized in the presence of DRB and TRB also shows sRNA and large molecular weight RNA. Autoradiography shows that in the young larvae as well a high proportion of the RNA synthesized in the presence of DRB and TRB is nucleolar.

From the antagonistic action of DRB and TRB (*see* Experimental), a direct degradation of RNA would not be anticipated. It remained possible that synthesis inhibition might result in secondary degradation. That this is not the case is shown in text-figure 3, where the chemicals have not altered the pattern of the RNA which was recently synthesized.

TEXT-FIGURE 3.—Stability of newly synthesized RNA in the presence of benzimidazoles. Control glands (117 pairs) were successively incubated: (a) in synthetic medium with uridine-³H (100 μ c/ml) for 45 minutes; (b) in medium with 220-fold excess of unlabeled uridine (1.0 mM) for 1 minute as a rinse; (c) in medium with uridine, as before, for 45 minutes, and (d) in fresh medium, as before, for 110 minutes. Other glands (124 pairs) received DRB and TRB (40 μ g/ml each) during (d). The different total counts between the experiments are mainly due to the different time of day at which they were run.



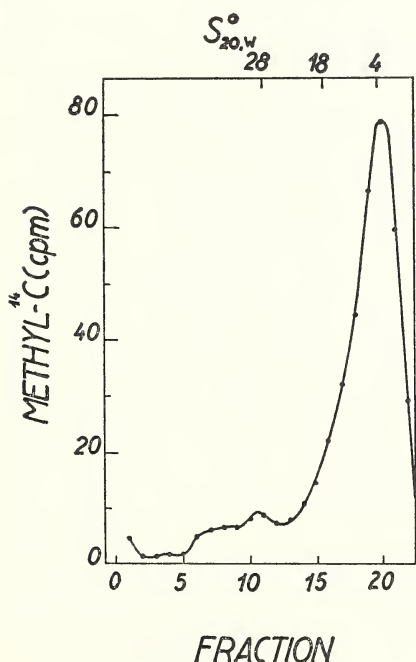
Synthesis of Nucleolar 4S RNA

It was previously found with autoradiography by use of methionine-(methyl- ^{14}C) that the nucleolus is the predominant cell site of RNA methylation (3). Whereas 90% of the nucleolar radioactivity was sensitive to ribonuclease, chromosomal and cytoplasmic radioactivities were not (4). Puromycin, which reduces the incorporation into protein, in fact, strongly reduces the considerable chromosomal and cytoplasmic incorporation only (3).

A sensitive criterion for RNA methylation was that the first appearance of radioactivity acquired from methionine(methyl- ^{14}C) coincided in its location at the central part of the nucleolus with the radioactivity derived from ^3H -nucleosides (3). Actinomycin, which inhibits nucleolar RNA synthesis almost selectively (17, 18), canceled the methylation as well. That the methylation occurs in the nucleolus itself is clearly indicated by its detection within 5 minutes of incubation with higher concentrations of tracer (150 $\mu\text{C}/\text{ml}$) and puromycin (0.7 mg/ml) than usual.

Nucleolar RNA methylation was examined in sedimentation gradient. Text-figure 4 shows that the methylation is mainly of 4S RNA, and that there is little in other RNAs. Thus, there is a correspondence between the cytochemical observations on methylation of nucleolar RNA and the methylation of 4S RNA which is nucleolar (text-fig. 1).

The profiles of RNA synthesis and methylation alter differently during gradual inhibition with actinomycin. Whereas at low doses of actinomycin the RNA accumulates at the top of the gradient and is reduced

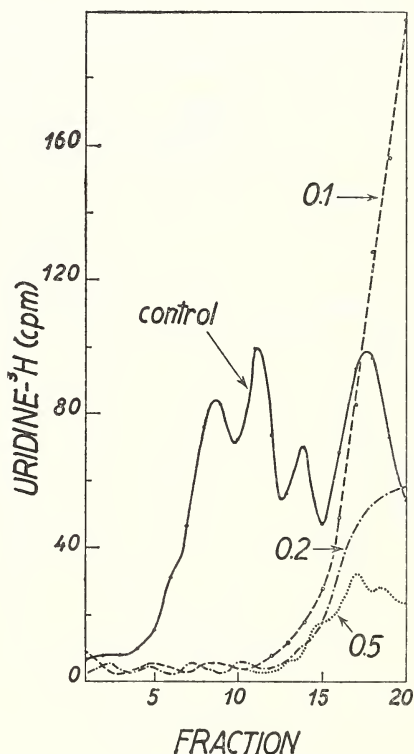


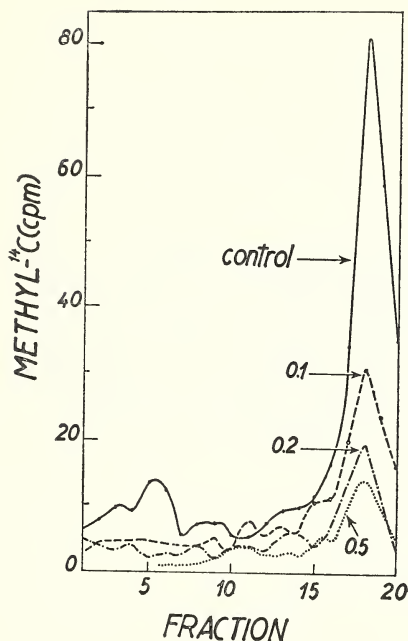
TEXT-FIGURE 4.—Methylation pattern of RNA in glands (169 pairs) incubated with methionine(methyl- ^{14}C) (50 $\mu\text{C}/\text{ml}$) for 30 minutes.

to a broad peak at high doses (text-fig. 5), the methylation decreases quantitatively and more rapidly at high doses (text-fig. 6). The inhibition pattern of synthesis strongly suggests degradation and/or synthesis of incomplete molecules of RNA, and this is supported by the enhanced cytoplasmic radioactivity concurrent with greatly reduced nuclear radioactivity observed with autoradiography. The inhibition of methylation indicates the rapid exhaustion of an RNA pool immediately available for methylation, and confirms that little of the RNA which remains in the upper (right) region of the gradient in text-figure 5 is 4S RNA capable of accepting methylation. These observations correspond closely with the inhibition of nucleolar synthesis and methylation found with autoradiography. Thus, the methylation of nucleolar RNA is immediately dependent on its synthesis.

Because of the immediacy between synthesis and methylation, it was possible to examine their correspondence directly. Glands were simultaneously treated with benzimidazoles, as in text-figure 1, so as to allow only nucleolar synthesis and to conditions of methylation as in text-figure 4. The result of this experiment (text-fig. 7) shows that the nucleolar 4S RNA is the true acceptor of methylation, since its degree of methylation remains identical when synthesis of other RNAs is severely reduced.

TEXT-FIGURE 5.—Inhibition of RNA synthesis by actinomycin. Glands were incubated as the control in text-figure 1 and in the presence of actinomycin. The control and glands treated with 0.1 and 0.2 $\mu\text{g}/\text{ml}$ actinomycin were incubated on the same day, and the respective counts from 98 and 170 pairs of treated glands were converted to 82 pairs in the control. Two hundred pairs were incubated with 0.5 μg actinomycin together with their own control on a different day, and the counts were recalculated for the present control on the basis of the observed percent inhibition. At high doses of actinomycin many nucleoli are destroyed.





TEXT-FIGURE 6.—Inhibition of RNA methylation by actinomycin. Control glands were incubated in synthetic medium for 110 minutes and then with methionine(methyl- ^{14}C) ($50\text{ }\mu\text{c/ml}$) for 45 minutes. Treated glands were incubated in the presence of actinomycin. The experiments with 0.1 and $0.2\text{ }\mu\text{g/ml}$ actinomycin and the control were run on the same day, and the respective counts from 99 and 151 pairs of treated glands were adjusted to 76 pairs in the control. The $0.5\text{ }\mu\text{g}$ actinomycin experiment was run on a different day with a control, and the counts for 167 pairs were adjusted to the present control.

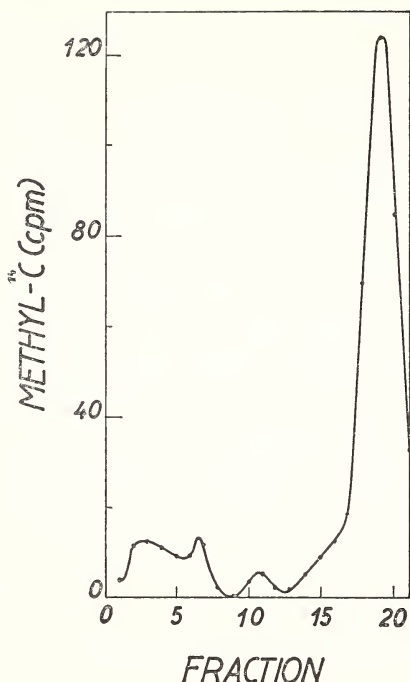
DISCUSSION

The main conclusion is that 4S and $> 28\text{S}$ RNA are synthesized in the nucleolus. This synthesis is present during a larval period (stage I to III) in which the nucleolus doubles its size (5). Throughout this period the gland is very active in the synthesis of protein (3, 6) and secretion (9).

Most of the evidence concerns the *de novo* synthesis of 4S RNA and will be summarily discussed as to: (a) the nucleolus is the site of synthesis, and (b) the RNA is a native molecule which (c) includes transfer RNA.

(a) The localization in the nucleolus of newly synthesized 4S RNA (text-fig. 1) is based on chemical isolation of the nucleolar synthesis, which has been validated quantitatively (table 1). A quantitative cytochemical test (3, 4) shows that RNA methylation is mainly nucleolar. The methylation occurs within 5 minutes and therefore most probably *in situ*, contrary to what is claimed for a different system (19). Because methylation is known to occur mainly to 4S RNA (text-fig. 4), this evidence points also to the nucleolar RNA being 4S. Similarly, actinomycin suppresses simultaneously the nucleolar RNA synthesis (17, 18) and nucleolar RNA methylation (3), and the synthesis and methylation of 4S RNA (text-figs. 5 and 6).

That the RNA is synthesized, rather than collected, at the nucleolus is favored by kinetic (5) and inhibition (13) studies, which also tend to exclude the nucleolus organizer as the site of synthesis. Recent observa-



TEXT-FIGURE 7.—Methylation of RNA during inhibition of non-nucleolar RNA synthesis. Glands (261 pairs) were incubated with benzimidazoles, as in text-figure 1, and then with methionine- ^{14}C , as in text-figure 4, in the presence of benzimidazoles.

tions suggesting the presence of DNA *inside* the nucleolus of *Smittia* (20) would provide evidence for the required internal primer.

(b) The native character of the nucleolar 4S RNA isolated by means of benzimidazoles is supported by the absence of degradation by these agents on recently synthesized RNA (text-fig. 3). The chemical procedure eliminates nuclease activity (*see* Experimental). In the normal cell, methylation is almost exclusively to 4S RNA (text-fig. 4). Since this is also observed during inhibition of most other RNA synthesis, or of practically *all* synthesis (text-fig. 6), it may be stated, on the basis that methylation follows closely after synthesis (text-fig. 6), that the RNA is not derivative. Further indication on the native character comes from section (c), ascribing a physiological role to the RNA.

(c) That the nucleolar 4S RNA contains transfer RNA can be inferred from several lines of evidence. It contains ribothymine (4) and other methylated bases (3) typical of transfer RNA. It was shown directly to be acceptor of methylation (text-fig. 7), and this is dependent on synthesis as shown directly (text-figs. 5 and 6) and by the recovery of uridine- ^{14}C as ribothymidine (4). The pool of still non-methylated RNA seems therefore short-lived. Precise characterization of the RNA is under way.

The coexisting syntheses of different RNAs raise the question of the relative disposition of the governing cistrons within the nucleolus. According to work on the viable divisibility of the nucleolus in *Chironomus* (21), the cistrons for the different RNAs would all have to be present at both ends of the nucleolus along the chromosomal axis.

Note added in proof: The recovery of radioactive RNA in autoradiographs prepared of squashes (table 1) was examined by high voltage electrophoresis analysis of all preparative solutions and of squashed glands at the stage when they are subjected to autoradiography. Model tests conducted with carrier yeast RNA alone revealed inappreciable analytical losses; tests with yeast RNA and purified *E. coli* transfer RNA showed no significant RNA degradation by acetic acid present during the squash routine and recovery analysis. In untreated glands labeled as in table 1, the RNA radioactivity in the glands was 98% of the total RNA radioactivity, and 98% of it was in UMP. The recovery of RNA was independently assessed on sections of glands treated with DRB-TRB and labeled as in table 1, showing in autoradiographs the same preponderant nucleolar RNA radioactivity as the squashed glands. In parallel experiments, after an adequate A_{260} input of gland RNA, no appreciable loss of total RNA during the entire histological procedure up to autoradiography was revealed with the use of spectrophotometry. These results validate the assignation to the nucleolus of the radioactive RNA in chemical experiments, where the RNA was extracted from glands in which nucleolar synthesis or methylation had been established with autoradiography (in press, Inter J Rad Biol, 1966).

Polyacrylamide gel electrophoresis was used to analyze further the nucleolar RNA labeled in DRB-TRB-treated glands with uridine- ^3H or in untreated glands with methionine(methyl- ^{14}C). This technique permits us to separate transfer RNA from both 5S ribosomal RNA and <4S RNA components. The ^3H -RNA profile revealed the presence, together with transfer RNA (and >28S RNA), of some 5S ribosomal RNA which is probably also of nucleolar origin. The methylation profile coincided entirely with transfer RNA.

RESUMEN

En las células salivares larvarias de un quironómido la síntesis nucleolar, aislada dentro de la célula por medios químicos, consiste de ARMs >28 y 4S.

REFERENCES

- (1) PERRY, R. P.: On ribosome biogenesis. Nat Cancer Inst Monogr 23: 527-545, 1966.
- (2) JACOB, J., BIRNSTIEL, M. L., and SIRLIN, J. L.: Nucleolar ribonucleic acid. In Nucleic Acid-Structure, Biosynthesis and Function. New Delhi, Council of Scientific and Industrial Research, 1965, pp 197-209.
- (3) SIRLIN, J. L., JACOB, J., and TANDLER, C. J.: Transfer of the methyl group of methionine to nucleolar ribonucleic acid. Biochem J 89: 447-452, 1963.
- (4) BIRNSTIEL, M. L., JACOB, J., and SIRLIN, J. L.: Analysis of nucleolar RNA synthesis in dipteran salivary glands. In Symposium on Molecular Cytology. Arch Biol (Liège) 76: 565-569, 1965.
- (5) SIRLIN, J. L., KATO, K., and JONES, K. W.: Synthesis of ribonucleic acid in the nucleolus. Biochim Biophys Acta 48: 421-423, 1961.
- (6) SIRLIN, J. L.: Cell sites of RNA and protein syntheses in the salivary gland of *Smittia* (Chironomidae). Exp Cell Res 19: 177-180, 1960.
- (7) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. IV. The nucleus of *Smittia parthenogenetica* (Chironomidae) with special reference to the nucleolus and the effects of actinomycin thereon. J Ultrastruct Res 11: 315-328, 1964.
- (8) ———: Electron microscope autoradiography of the nucleolus of insect salivary gland cells. Nature (London) 202: 622-623, 1964.

- (9) KATO, K.-I., and SIRLIN, J. L.: Aspects of mucopolysaccharide production in larval insect salivary cells. A combined cytochemical-autoradiographical approach. *J Histochem Cytochem* 11: 163-168, 1963.
- (10) KROEGER, H.: Zellphysiologische Mechanismen bei der Regulation von Genaktivitäten in den Riesenchromosomen von *Chironomus thummi*. *Chromosoma* 15: 36-70, 1964.
- (11) SIRLIN, J. L., TANDLER, C. J., and JACOB, J.: The relationship between the nucleolus organizer and nucleolar RNA. *Exp Cell Res* 31: 611-615, 1963.
- (12) SIRLIN, J. L., JACOB, J., and BIRNSTIEL, M. L.: Synthesis of different species of nucleolar ribonucleic acid. *Biochim Biophys Acta* 108: 716-718, 1965.
- (13) SIRLIN, J. L., and JACOB, J.: Sequential and reversible inhibition of synthesis of ribonucleic acid in the nucleolus and chromosomes: effect of benzamide and substituted benzimidazoles on dipteran salivary glands. *Nature (London)* 204: 545-547, 1964.
- (14) ALLFREY, V. G., MIRSKY, A. E., and OSAWA, S.: Protein synthesis in isolated cell nuclei. *J Gen Physiol* 40: 451-490, 1957.
- (15) GEUSKENS, M.: Etude autoradiographique de l'effet des inhibiteurs métaboliques sur les relations entre le noyau et le cytoplasme des oocytes d'astéries et de grenouilles. *Arch Biol (Liege)* 72: 153-171, 1961.
- (16) NAKAMOTO, T., FOX, C. F., and WEISS, S. B.: Enzymatic synthesis of ribonucleic acid. I. Preparation of RNA polymerase from extracts of *Micrococcus lysodeikticus*. *J Biol Chem* 239: 167-174, 1964.
- (17) SIRLIN, J. L., and JACOB, J.: Function, development and evolution of the nucleolus. *Nature (London)* 195: 114-117, 1962.
- (18) SIRLIN, J. L., JACOB, J., and KATO, K.-I.: The relation of messenger to nucleolar RNA. *Exp Cell Res* 27: 355-359, 1962.
- (19) COMB, D. G., and KATZ, S.: Studies on the biosynthesis and methylation of transfer RNA. *J Molec Biol* 8: 790-800, 1964.
- (20) JACOB, J.: Intr nucleolar deoxyribonucleic acid components in insect cells as revealed by electron microscopy. *Nature (London)* 211: 36-38, 1966.
- (21) BEERMANN, W.: Der Nukleolus als lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.

DISCUSSION

Perry: As you know, we did very similar experiments on mammalian cells in tissue cultures in which, at low doses of actinomycin D where the nucleolar synthesis is inhibited down to about 20% of normal, we find an incorporation of precursor into 4S RNA which is not significantly different from that found in untreated cells (Perry, *Proc Nat Acad Sci USA* 48: 2179-2186, 1962). We measured the base compositions of this 4S RNA made in the presence of actinomycin, and it is identical to the 4S RNA made in control cells (Perry, *Nat Cancer Inst Monogr* 18: 325-340, 1965). We also tested for the presence of terminal labeling by observing the kinetics of degradation induced by snake venom diesterase, and we found that there is no appreciable terminal labeling under the conditions of our experiments (Perry, *Nat Cancer Inst Monogr* 14: 73-89, 1964). Therefore it would seem that our experiments are somewhat in contradiction to yours, although one could always argue that they are different systems and possibly the mammalian cells would give one result and the insects would give another. One possibility that occurs to me, however, is that in our case of actinomycin inhibition the 20% of activity which remains, and can be counted as nucleolar, is mainly on the periphery of the nucleoli. In your case is there any evidence that the 4S RNA which you see is distributed slightly differently than normal? That is, since in your system the nucleolar organizer is in the center of the nucleolus, and since there is other DNA and obviously other cistrons outside,

would it be possible that the 4S RNA is synthesized on cistrons close to the nucleolar organizer but not sufficiently close to be the same?

Sirlin: All I can say at this time is that when we look at the nucleolar radioactivity after 45 minutes of incubation, which is what goes into the sucrose gradient, most of the radioactivity hasn't reached halfway toward the nucleolar periphery. Of course, one always sees a few grains in the rest of the nucleolus. In the chromosome, with the inhibitors present, which is how we detect the nucleolar 4S RNA, there is hardly any activity in the organizer or in the entire chromosome. Of course, from the actinomycin experiments in our cells I can say nothing because there is practically no true 4S RNA left, as judged by methylation, and most of the time at the high doses not even a nucleolus left. In your cells, composition alone may be insufficient to characterize 4S RNA which resembles closely the other RNAs.

Perry: In the beginning of your talk, when you said you were settling the controversy between *Chironomus* and *Smittia*, didn't you say that the earlier results showed the labeling was not close to the center of the chromosome but indeed in the periphery, and this was what early led you to believe it was RNA primed?

Sirlin: The peripheral labeling was in *Drosophila* and *Rhynchosciara*. In *Smittia* we have always maintained that at early nucleolar labeling, before there is any chromosomal label, you never see radioactivity in what can be called the bulk or core of the organizer proper. We have checked this with electron microscope autoradiography. I don't believe the Beermann school (Pelling, *Chromosoma* 15: 71-122, 1964) has gone so thoroughly into this as we have. We have always said that the synthesis starts in the nucleolus proper close to what, at the light microscope level or even with electron microscopy, you can recognize as that part of the nucleolus chromosome going through the nucleolus.

Perry: And then moves in?

Sirlin: And then moves out. It never moves in. Of course, by then the chromosomes themselves start getting labeled and the organizer itself too. In everything we have done, all sorts of innovations, we have looked at this very closely, putting the cell into very strained situations, such as starting or rapidly increasing synthesis, or cutting it off completely. We have done this with a compound called tricyamp (Jacob and Sirlin, *Science* 144: 1011-1012, 1964) and we published similar experiments in *Nature* [Sirlin and Jacob, *Nature* (London) 204: 545-547, 1964] using the benzimidazoles. In all cases the organizer region of the chromosome plays ball with the rest of the chromosome, but in no way plays ball with the nucleolus proper, and it labels later than the nucleolus.

Swift: I would like to ask you a question concerning heterochromatin and the nucleolar organizer. In *Sciara* and many other forms (possibly also in *Smittia*, although we have never studied that species) the nucleolus organizer locus is associated with densely Feulgen-positive material. This chromosomal region stays condensed even when a large nucleolus is present, and we have never been able to detect within it the presence of RNA. I assume this region may be considered "nucleolus organizer-associated chromatin," rather than the organizer itself, and I wonder if the situation in *Smittia* is not similar; in other words, there is inactive chromatin on either side of the organizer, while the organizer region itself is diffusely expanded within the nucleolar substance.

I would like to ask a second question: Do you think it is possible that transfer RNA might occur within the nucleolus, and yet not be made at the organizer locus? Could the nucleolus be a "sink" that absorbs RNA made elsewhere in the nucleus?

Sirlin: To take the second point first, I think the collection of RNA from elsewhere in the nucleus into our nucleolus can be discarded absolutely for any stage. At least I won't budge on this point until someone comes along and shows in my own system that I am wrong. Now, about the discrepancy that you don't find any DNA threads at the periphery where you do find labeled RNA. Well, we can see beautiful knobs of DNA throughout, but we cannot see any real accumulation of them at the site where RNA synthesis is first observed, so I am assuming there

are interconnecting DNA filaments there, but these remain to be demonstrated. In the same vein, in your system you perhaps still have some filamentous DNA extending to the periphery that you cannot observe. Of course, this is simply a proposal. Now as to the first question, I'm not certain I understood what you were asking me. Morphology is difficult to put in words sometimes.

Swift: My first question concerned the nucleolus organizer. I believe you have concluded that, since you see no nucleolar label over the chromosome in the immediate region of the nucleolus, that nucleolar RNA may not be synthesized on a chromosomal DNA template. In the *Sciara* with which we are familiar, there is a region of strong DNA stainability which does not contain noticeable RNA. I think this is a heterochromatic region adjacent to the organizer, not the organizer itself. I would not expect to find any tritiated uridine label in a region where cytochemically you cannot detect any RNA.

Sirlin: The situation with the organizer in *Smittia*, as observed with the electron microscope, is as follows [see accompanying fig. from (7)]. There are three parts of the nucleolus in *Smittia*. The proximal portion (PP) is finely particulate and in sections can be seen as knobs (NM) interspersed through the chromosomal DNA. So this zone goes through and around the DNA band or DNA bands at this region. Next there is a large intermediate amorphous region (IP) and then a very thin, gross particulate region (OCR). Now synthesis starts at PP, and perhaps at the nearby IP (20). I'm not trying to commit ourselves whether it starts there rather than at the very proximal edge of PP. We haven't done enough of this. As to the shortage of evidence, you must admit that it is very difficult to pick an electron micrograph of this region which demonstrates the entire field and have it be an early labeling autoradiograph as well. As far as we can tell, the radioactivity in the nucleolar knobs (NM) inside the organizer appears at about the same time as the radioactivity in the body of the nucleolus (PP), and there is no labeling in the organizer or elsewhere in the chromosome at that time. As we now know, in PP there may be interconnecting DNA filaments acting as templates, but whether they represent a different intranucleolar organizer as you suggest remains to be seen.

Pavan: In *Rhynchosciara angelae* the nucleolar organizer region is located at the base of the X chromosome. When a nucleolus is formed, one can very frequently see DNA fibers dispersed inside it. In some cases part of these fibers form a typical chromomere which lies in a more or less central region of the nucleolus. Beside this, there are several bands near the nucleolar organizer region that sometimes are condensed and at other times dispersed.

Another point: In *R. angelae* we have shown that the normal dispersed nucleolus existing in the nucleus of cells of the salivary gland can be transformed into a typical spheroidal nucleolus if the salivary gland is left for a half hour or more in Ringer. Dr. Sirlin, did you inject precursor into the larvae to see if the results *in vivo* are similar to the ones you get *in vitro*?

Sirlin: Yes, I did. In fact, the beginning of my work many years ago consisted of injecting larvae *in vivo*. We switched over to the *in vitro* system because it is the only way one can do inhibition experiments and be sure that what is offered gets to the gland and is not metabolized by the rest of the larva.

Pelling: I should like to ask you first whether you have done RNA extraction experiments on the fixed tissue which you have used for autoradiographs? Are you sure that the 4S RNA is still present in your autoradiographs? Second, it seems unlikely to me that the nucleolar organizers of *Chironomus* and *Smittia* should behave differently. Puffing should also occur in your material. I would expect you to find patterns of DNA labeling often extending throughout the nucleolus, if you use thymidine with high specific activity and long exposure times. It would be adequate to label the DNA in the presence of the inhibitors. Third, what do you think about the possibility that the inhibitors prevent the transition of the high molecular weight nucleolar RNA (>28S) into the 28S and 18S RNA fractions? This might explain,

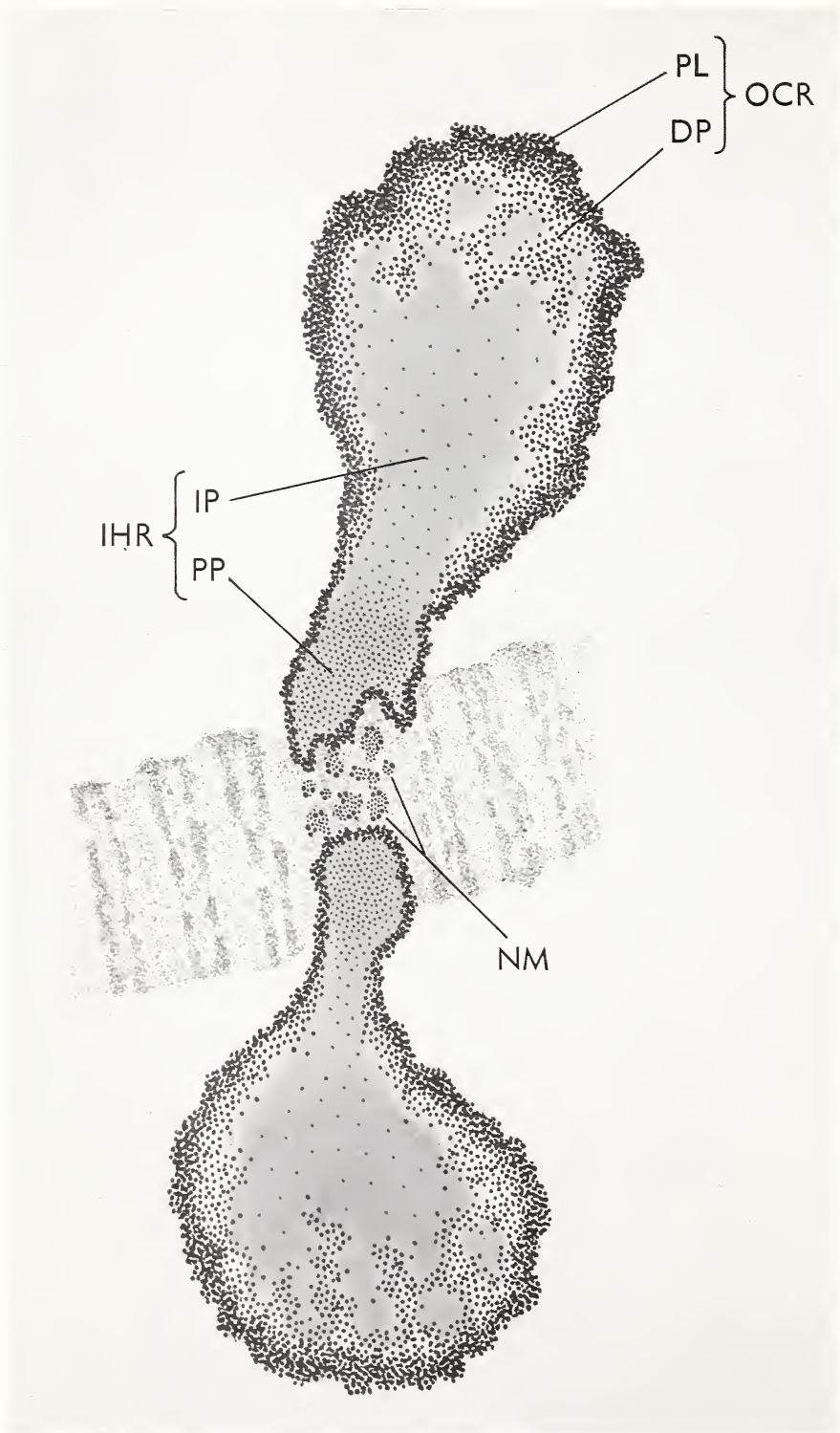
at least partly, the relative intensity of the nucleolar label and the fact that you did not find 28S and 18S RNA label.

Sirlin: Regarding the first comment on quantitative recovery in the autoradiography, this is being checked chemically at the moment. The glands being prepared for autoradiography stand for 1 hour in 5 percent TCA at ice temperature. This is a very strong fixation, and thereafter they go only briefly through distilled water. To be quite fair, the only tricky step here is the acetic acid that one uses for squashing, and we keep it at about -5°C for 40 seconds. All this is done in the presence of a very high concentration of lanthanum which is, I believe, a very strong precipitant. This is all I can tell you at the moment, but I can promise you that this test is being done.

But that our autoradiographical pattern results from loss of 4S RNA, I believe is wrong. I believe this because, when we used lower concentrations of tracer and lower specific activity, the only peak apparent was the 4S and the only radioactivity was nucleolar [Jacob, Birnstiel, and Sirlin, Council of Scientific and Industrial Research, New Delhi, p 107, 1965; Birnstiel, Jacob, and Sirlin, Arch Biol (Liège) 76: 565-589, 1965]. And what is crucial, the methylation, which is practically all in 4S RNA in the gradient, is unequivocally correlated with the nucleolar RNA methylation, which is the only one found in the cell by autoradiography.

As to the cytology of the organizer, I'm not going by my experience at all because I don't consider myself a cytologist. But I asked Bauer (unpublished observations) for his best photographs showing puffing of the organizer in this species, and all he could produce, apart from having told this to me several times in conversation, were tiny branches coming out of the organizer, which you can also see in our long incubations and exposures with thymidine (11). As to the prevented transition from precursor to ribosomal RNAs, this is possible.

Barr: I want to point out first that there is a paper by Dr. Plaut and Dr. Nash of Wisconsin (Nash and Plaut, J Cell Biol 27: 682-686, 1965) describing DNA particles throughout the nucleolus of the salivary gland cells of *Drosophila melanogaster*; and second, that I have, working with Dr. Plaut in his laboratory, recently extended this to other species of *Drosophila* and find that the structural details are different from species to species. I should like to make a plea for people to realize that this is not merely a set of morphological curiosities, but is very relevant to the important functional problem of whether this DNA is or is not attached to the chromosomes and whether it ever has been attached to the chromosomes. It has been assumed by many people, by Dr. Pavan, often by myself, and by others, that small particles of DNA found within the nucleolus must be attached somehow to the chromosomes. I would like to suggest, however, that Dr. Miller's extremely important findings (this volume) may be relevant here in that what we are seeing may be an amplification of certain chromosomal regions. We may be seeing DNA that has come off the chromosome, only here in *Drosophila* it all stays together in one nucleolus instead of breaking up into separate ones as in the newt oocyte. All of which finally leads us to remember that Painter, who first described the cytogenetical importance of *Drosophila* salivaries and also first claimed to show Feulgen-positive particles in amphibian micronucleoli, originally had this idea and suggested that so-called heterochromatin may actually be chromatin material which is being rapidly synthesized for discharge from the chromosomes.



Autoradiographic Studies of Transfer RNA Metabolism in *Vicia faba* Root-Tip Cells^{1, 2}

PHILIP S. WOODS, *Department of Biological Sciences,
University of Delaware, Newark, Delaware*

SUMMARY

Parallel biochemical and autoradiographic experiments were performed on growing *Vicia faba* meristems. A highly specific extraction procedure was developed which quantitatively removed most of the transfer RNA (tRNA) from frozen ethanol-substituted and fixed tissue without removing other forms of RNA or DNA. When this procedure was applied to sections of H³-cytidine-treated roots, autoradiography of tRNA alone

was possible. Grain-counted data indicated: 1) some tRNA was first synthesized in the chromatin and later diffused to the cytoplasm; 2) the nucleolus contained no measurable tRNA, and 3) some tRNA was either synthesized in the cytoplasm or, what is more likely, the . . . cytosine-cytosine-adenine end of the molecule was attached in the cytoplasm.—*Nat Cancer Inst Monogr* 23: 271–284, 1966.

IT HAS been known for some time that soluble or transfer RNA (tRNA) plays a central role in the organization of amino acids to form polypeptide chains during protein synthesis. Individual tRNA molecules combine with a particular amino acid to produce a complex active on the ribosomal template. Clearly, therefore, tRNA occurs in the cytoplasm of most cells. Recent annealing experiments (1,2) make it likely that for some time tRNA or its precursors exist in the nucleus, more specifically, in association with the chromosomal DNA when the precursor molecules are synthesized. Other experiments (3–5) suggest that tRNA-like molecules occur also in the nucleolus. This widespread distribution of tRNA and its precursors in the cell demonstrates the complexities associated with the synthesis of these molecules, and the resulting confusion has prompted us to study tRNA metabolism autoradiographically. Our approach has been to use the labeled and general nucleic acid precursor H³-cytidine in combination with a differential extraction method that might selectively remove tRNA from sections of fixed cells without disturbing the arrange-

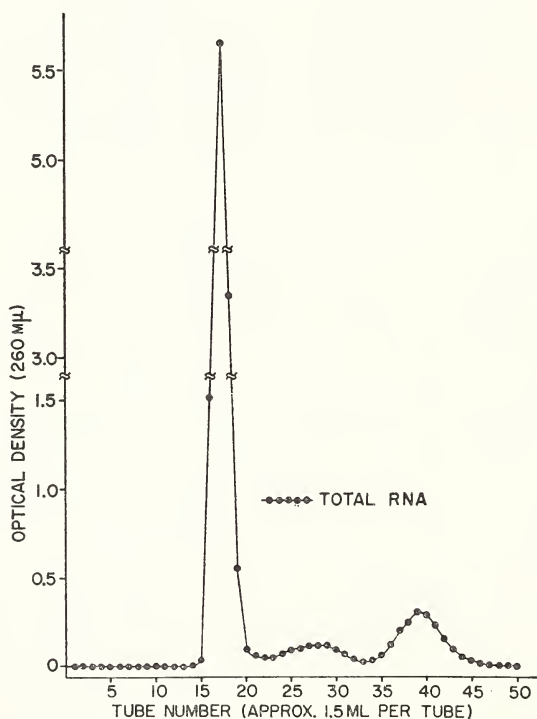
¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Research carried out at the University of Delaware under contract AT(30-1) 2939 with the U.S. Atomic Energy Commission.

ment of other forms of RNA or DNA. It would seem likely that tRNA could be followed by measurement of the uptake of H^3 -cytidine into the nucleic acids of the various cellular compartments, the difference in these values, before and after extraction, being used as a measure of the concentration of labeled tRNA in each compartment. This approach has proved successful in our laboratory (6), and the present report will describe these experiments and summarize our findings.

BIOCHEMICAL STUDIES: DIFFERENTIAL EXTRACTION OF tRNA FROM FIXED TISSUE POWDER

If 20 g of frozen *Vicia faba* meristems are ground in a coffee mill to a fine powder in liquid nitrogen, thawed briefly by suspension in water, and mixed and shaken for a few hours with equal volumes of freshly distilled phenol, a relatively pure extract of the total RNA's of the tissue can be obtained. This extract contains less than 2% DNA. When this solution is analyzed by elution on a Sephadex G-100 column (text-fig. 1), three peaks of RNA occur (6). Since Sephadex generally fractionates



TEXT-FIGURE 1.—Sephadex gel filtration (6) of a 2 ml aliquot of total RNA extract (phenol) obtained from 20 g of frozen *Vicia faba* meristems. At *left* are faster moving RNA components and hence those of greater molecular size. Components to *right* are smaller in molecular size.

according to the size of the molecule, with the first substance eluted corresponding to the largest molecules and the last eluted to the smallest, evidently these peaks represent classes of RNA differing from one another in regard to molecular weight. If the classes are then tested for their ability to form a complex (aminoacyl tRNA) with C^{14} -leucine, it would be expected that tRNA could be detected, since only this type of RNA can form complexes with an amino acid. Table 1 shows that only the third peak, the smallest molecular-sized class, forms a complex. Clearly, therefore, this peak represents tRNA. The other two classes have not been further characterized; however, judging from the relative amounts (from left to right: 81, 6, and 13%) and nature of the peaks, possibly the largest molecular-sized class corresponds to ribosomal RNA, and the intermediate molecular-sized class to polydispersed messenger RNA. Further work on the characterization of these peaks is in progress.

For the purpose of the present study, we were interested in developing an extraction technique that would selectively remove from fixed tissue the RNA of the third peak (tRNA) without removing the RNA of the other two peaks (ribosomal and possibly messenger RNA). This was accomplished, as the following experiment illustrates. When 20 g of frozen, ethanol-substituted, and fixed (67% ethanol plus 0.67% KAc, 2 C for 90 min) tissue powder was treated for 30 minutes at 2 C with an extracting mixture consisting of 0.01 M $MgCl_2$, 0.3 M NaCl, and 0.001 M Tris buffer adjusted to pH 7.4 with HCl, up to 87% of the total tRNA of the tissue could be extracted without removal of the other types of RNA or DNA. Text-figure 2 illustrates this and shows the results when a two-step extraction process was applied to the ethanol-substituted and fixed tissue powder: 1) In the first step the fixed tissue powder was differentially extracted with the solution mentioned above. This RNA (*open circles*) showed the same elution pattern as the third peak in text-figure 1; also, these two RNA's had the same capacity for combining with leucine (table 1). 2) After the differentially extracted RNA was removed, a second step, resuspending and shaking the tissue residue in 50% aqueous phenol, isolated the residual

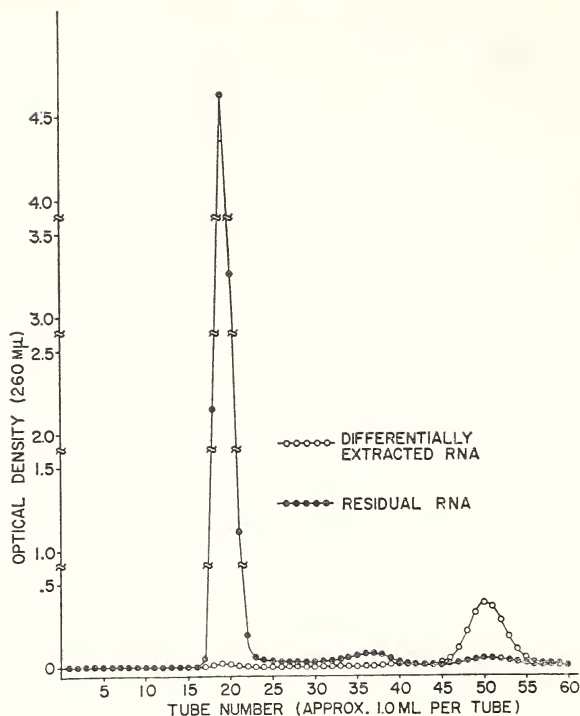
TABLE 1.—Comparison of various RNA fractions for their ability to form a complex (aminoacyl tRNA) with C^{14} -leucine*

Fraction studied	Counts/minute†/ OD unit
Tubes 33 through 46 of experiment shown in text-figure 1.....	161
Tubes 15 through 32 of experiment shown in text-figure 1.....	2
Differentially extracted RNA shown in text-figure 2.....	153
Residual RNA shown in text-figure 2.....	10

*Complex formation determined according to Woods and Zubay (6).

†Measured with a Packard Tri-Carb Liquid Scintillation Counter, using the scintillation mixture of Bray (Bray, G. A., Anal Biochem 1: 279, 1960).

THE NUCLEOLUS



TEXT-FIGURE 2.—Sephadex gel filtration of a 2 ml aliquot of differentially extracted RNA (soluble in 0.01 M MgCl, 0.3 M NaCl, 0.001 M Tris buffer pH 7.4) and a 2 ml aliquot of residual RNA (not soluble in above but extractable with phenol) from 20 g of frozen ethanol-substituted and fixed *Vicia faba* meristems.

RNA's. In text-figure 2, the elution pattern of the residual RNA's (closed circles) shows the first two peaks in the same relative amounts as in text-figure 1 but with the almost complete absence of the third peak. Evidently the extraction technique is highly specific for the removal of only tRNA. DNA apparently is not removed, since the first extract contains less than 2% DNA as determined by the Dische diphenylamine reaction (6).

AUTORADIOGRAPHIC STUDIES OF UNEXTRACTED AND EXTRACTED SECTIONS OF H^3 -CYTIDINE-TREATED ROOTS

The extraction technique developed for tissue powder was extended to sections of similarly processed whole roots treated with H^3 -cytidine (4.0 μ c/ml; specific activity 2.5 c/mm) for varying periods of time. Details of the techniques have appeared elsewhere (6); however, a brief description of these follows. After isotope treatment, roots were processed by freeze ethanol-substitution, embedded in paraffin, and sectioned. The sections were attached in the dark to the outer surface of dry AR-10 stripping

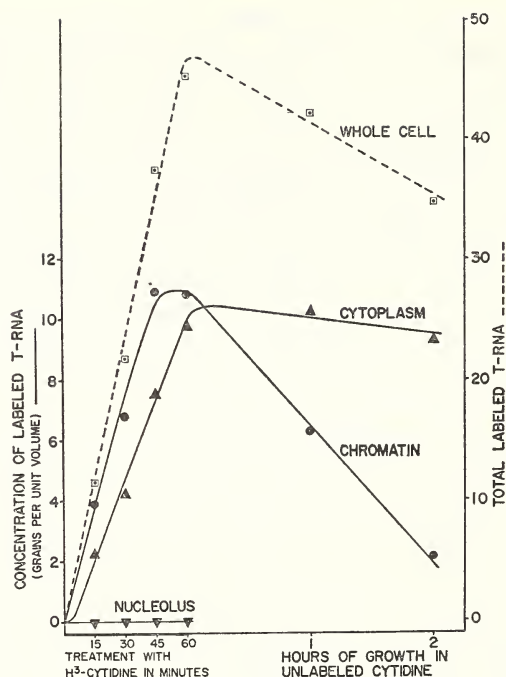
film, previously mounted on blank slides with sensitive side of emulsion up, by a dry-mount method. After the paraffin was removed, the sections were fixed (in the dark) as described for the tissue powder. Half of the slides were then removed from the fixative, rinsed in absolute ethanol, and air-dried, and the other half were treated in the same extracting mixture and under the same conditions as described for the tissue powder. These slides were also rinsed in absolute ethanol and air-dried. Thus unextracted and differentially extracted sections were obtained for each treatment period with H^3 -cytidine prior to exposure to the photographic emulsion. The preparations later were developed and the sections stained according to standard procedures. It is important to note that the fixation and differential extraction procedures described here for tissue sections are identical to the procedures described for tissue powder.

Figures 1 and 2 are examples of typical autoradiographs. Figure 1 shows autoradiographs of root cells treated with H^3 -cytidine for 1 hour, where (a) is of an unextracted section, and (b) is of a differentially extracted section. Reduction of radioactivity for the cytoplasm and chromatin is uniform after this treatment. No reduction appears for the nucleolus. Figure 2 shows autoradiographs of cells of a similarly treated root but where H^3 -cytidine treatment is followed by a 2-hour chase with a 1200-fold increase of unlabeled cytidine. The reduction of radioactivity is noticeable for the cytoplasm of the extracted section (fig. 2b) and very little reduction appears in other parts of the cell.

Quantitative data showing changes in concentration of presumably labeled tRNA during the experiments for the nucleolus, chromatin, and cytoplasm are summarized in text-figure 3. These data were obtained by subtraction of the concentration data for each structure in 10 cells of differentially extracted sections from corresponding data for 10 cells of unextracted sections, for each treatment period. Total labeled tRNA for whole cells, calculated from the concentration data, is also shown.

CONCLUSIONS AND DISCUSSION: SYNTHESIS AND TRANSLOCATION OF tRNA IN THE CELL

Since treatment of the tissues by the biochemical and autoradiograph methods was parallel, it can be assumed that the results of each are also parallel. The difference in the autoradiographs between the unextracted and extracted sections therefore serves as a measure of the uptake of H^3 -cytidine into the tRNA of each compartment. In the 1-hour-treated roots, the uniform reduction of radioactivity for the chromatin and cytoplasm (fig. 1), after differential extraction, indicates that labeled tRNA is present in these structures and not localized to any part of each structure. The nucleolus apparently contains no tRNA, since no reduction of radioactivity appears in this structure. After a 2-hour chase with cold cytidine



TEXT-FIGURE 3.—Incorporation of H^3 -cytidine into tRNA of various structures in cells of *Vicia faba* root tips. Ordinate at left refers to concentration of labeled tRNA (solid lines). Ordinate at right refers to total labeled tRNA for whole cells (dashed line). Left part of abscissa refers to increasing treatment time up to 60 minutes. Right part of abscissa refers to chase period in hours after the 60-minute treatment with H^3 -cytidine.

(fig. 2), uniform reduction of radioactivity by differential extraction is noticeable only for the cytoplasm and indicates the presence of tRNA only in this one compartment after this treatment.

From the summary of autoradiographic results presented in text-figure 3, it can be seen that the concentration of labeled tRNA in the chromatin rises rapidly for the first 45 minutes of treatment with H^3 -cytidine. The concentration of labeled tRNA in the cytoplasm rises at a lower linear rate and for the entire hour of labeling. No labeled tRNA appears in the nucleolus at any time. During the chase period the concentration of labeled tRNA for the chromatin declines rapidly, whereas that for the cytoplasm declines only slightly.

When the concentration values of labeled tRNA for the cytoplasm are converted to total labeled tRNA values³ and added to the corresponding concentration values for the chromatin, the average value for the total labeled tRNA of a cell for each treatment period may be calculated and

³ Since the volume of the cytoplasm of these cells is, on the average, 3.5 times that of the chromatin, the concentration values for the cytoplasm may be converted to total labeled tRNA values by multiplication of each by this factor.

plotted (*dashed line* in text-fig. 3). The amount of labeled tRNA rises linearly throughout the entire treatment period with H^3 -cytidine. However, at the beginning of the chase the rate of incorporation into tRNA shifts abruptly, and during the chase a distinct decline in amount of labeled tRNA per cell occurs. Such a decline might reflect the stability of the molecule in the cell.

These findings are consistent with the view that some tRNA is synthesized in the chromatin and that later it diffuses to the cytoplasm. However, if all the labeled cytoplasmic tRNA was accounted for by nuclear synthesis, an initial lag in the labeling pattern of the cytoplasm would be expected. On the contrary, the data indicate that cytoplasmic tRNA labeling appears almost immediately and increases linearly throughout the hour of treatment with H^3 -cytidine. An appreciable amount of cytoplasmic tRNA labeling must come from some pathway other than that of the nucleus. Possibly some *de novo* tRNA synthesis occurs in the cytoplasm. *A priori* it seems far more likely that the extra labeling is accounted for by the addition of cytidine residues to the end of the tRNA molecule at the time the terminal . . . cytosine-cytosine-adenine grouping is attached. In other systems the tRNA that invariably contains this nucleoside terminal grouping also shows addition of the respective nucleotides in a stepwise fashion (7). The enzyme incorporating the trinucleotide terminus into tRNA has been isolated (8) and is distinct from DNA-dependent RNA polymerase.

The lack of tRNA labeling in the nucleolus is very striking and apparently demonstrates that tRNA or its precursors do not exist in this structure. This observation contradicts the suggestion (3-5) that tRNA-like molecules exist in the nucleolus. Some investigators believe (3, 4) that the nucleolus is a center for the methylation of tRNA, while others (5, 9) offer evidence that methylation takes place in the cytoplasm on the ribosomal particles. Other experiments designed to test these possible nucleolar activities are in progress.

RESUMEN

Se realizaron experimentos bioquímicos y autorradiográficos paralelos en meristemas de Vicia faba en crecimiento. Se desarrolló un procedimiento de extracción altamente específico que extrae cuantitativamente la mayor parte del ARN-t a partir de tejido congelado sustituido por etanol y fijado sin extraerle otras formas de ARN o ADN.

Cuando se aplicó este procedimiento en cortes de raíces tratadas con citidina H^3 , fue posible autorradiografiar solamente el ARN-t. Los datos de recuentos de granos indicaron: (1) que algo de ARN-t se sintetiza primero en la cromatina y que después difunde al citoplasma; (2) que el nucleolo no contiene ARN-t medible; (3) que algún ARN-t se sintetiza en el citoplasma o que, lo cual es más probable, el extremo de la molécula—citosina-citosina-adenina se une en el citoplasma.

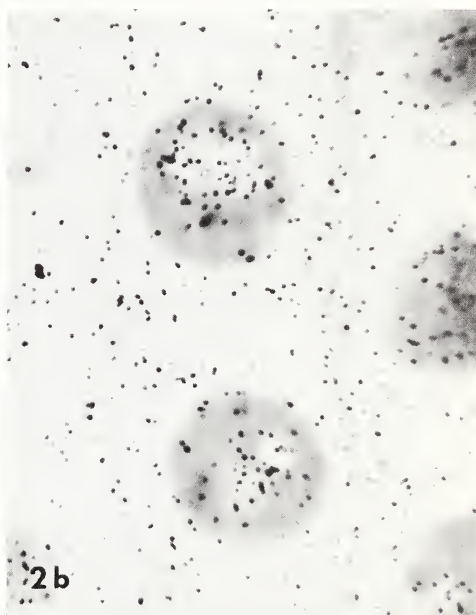
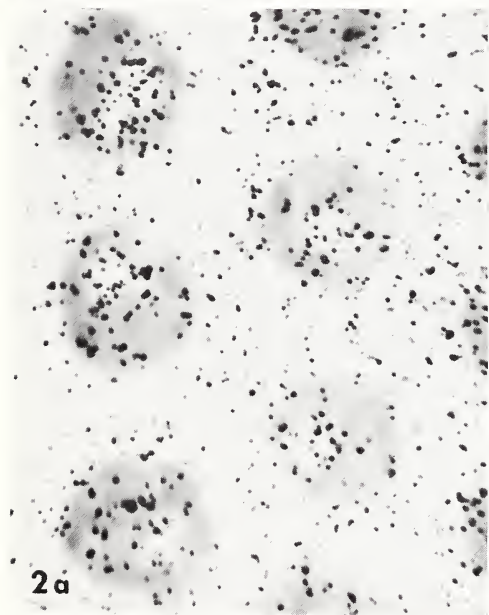
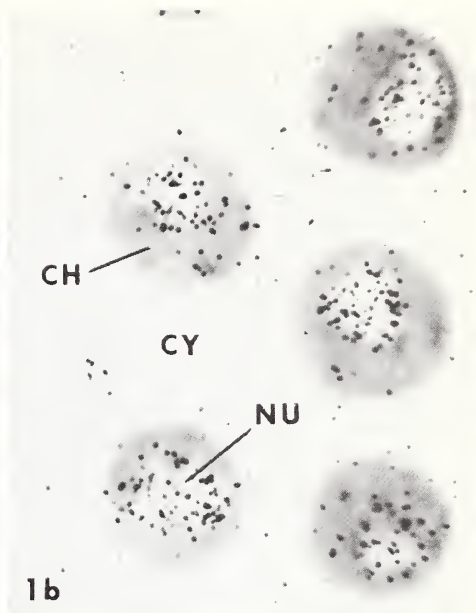
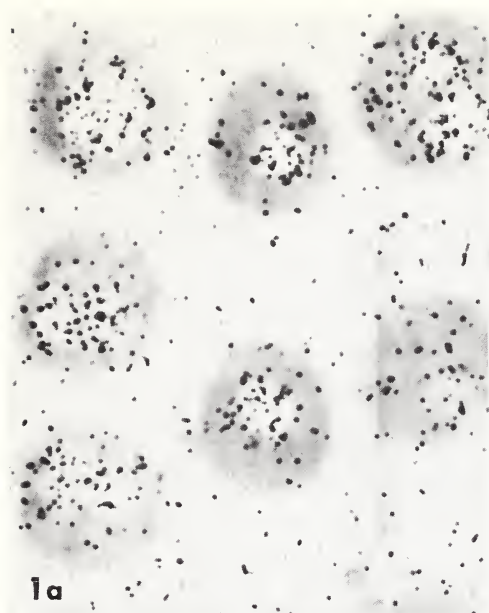
REFERENCES

- (1) GIACOMONI, D., and SPIEGELMAN, S.: Origin and biologic individuality of the genetic dictionary. *Science* 138: 1328-1331, 1962.
- (2) GOODMAN, H. M., and RICH, A.: Formation of a DNA-soluble RNA hybrid and its relation to the origin, evolution, and degeneracy of soluble RNA. *Proc Nat Acad Sci USA* 48: 2101-2109, 1962.
- (3) BIRNSTIEL, M. L., FLEISSNER, E., and BOREK, E.: Nucleolus: A center of RNA methylation. *Science* 142: 1577-1580, 1963.
- (4) SIRLIN, J. L., JACOB, J., and TANDLER, C. J.: Transfer of the methyl group of methionine to nucleolar ribonucleic acid. *Biochem J* 89: 447-452, 1963.
- (5) COMB, D. G., and KATZ, S.: Studies on the biosynthesis and methylation of transfer RNA. *J Molec Biol* 8: 790-800, 1964.
- (6) WOODS, P. S., and ZUBAY, G.: Biochemical and autoradiographic studies of different RNA's: Evidence that transfer RNA is chromosomal in origin. *Proc Nat Acad Sci USA* 54: 1705-1712, 1965.
- (7) HECHT, L. I., STEPHENSON, M. L., and ZAMECNIK, P. C.: Dependence of amino acid binding to soluble ribonucleic acid on cytidine triphosphate. *Biochim Biophys Acta* 29: 460-461, 1958.
- (8) CANELLAKIS, E. S., and HERBERT, E.: Studies on S-RNA synthesis. I. Purification and general characteristics of the RNA-enzyme complex. *Proc Nat Acad Sci USA* 46: 170-178, 1960.
- (9) GALIBERT, F., LARSEN, C. J., LELONG, J. C., and BOIRON, M.: RNA of low molecular weight in ribosomes of mammalian cells. *Nature (London)* 207: 1039-1041, 1965.

PLATES

PLATE 38

FIGURES 1 AND 2.—Autoradiographs of sections of *Vicia faba* root-tip cells stained after exposure and development by a ribonuclease-toluidine blue technique. All exposures were 15 days. Figure 1: Cells of root treated with H^3 -cytidine for 60 minutes (*a*) unextracted, and (*b*) differentially extracted with 0.01 M $MgCl_2$, 0.3 M NaCl, 0.001 M Tris buffer pH 7.4 at 2 C for 30 minutes. Note marked reduction of radioactivity in cytoplasm (CY), slight reduction in chromatin (CH), and no reduction in nucleoli (NU) of extracted section. Figure 2: Cells of root treated with H^3 -cytidine for 60 minutes followed with a 2-hour chase with medium containing excess unlabeled cytidine. (*a*) Unextracted, (*b*) extracted as in figure 1b. Note slight reduction of radioactivity in cytoplasm, and little or no reduction in chromatin and nucleoli of extracted section.



DISCUSSION

Sirlin: There are several points I would like to make. First, how much H^3 -cytidine or uridine is left in your frozen sections? These nucleosides, I would say, would have disappeared in your column. They would have separated from the RNA. I am extremely skeptical that all of the unincorporated cytidine is removed from your frozen sections. In our experience, even though we use more drastic procedures of cleaning the material than you do, we still find uridine together with the RNA until we get to the phenol step. I am trying to say that the correlation between your column determinations and the counts from the autoradiographs may be extremely weak. In fact, you have used Carnoy's fixative in the past and I believe the picture there was completely different: Very little label was in the chromatin region. I remember also that a few years ago Edström considered the possibility of loss of RNA from sections of Carnoy-fixed tissue (Edström, Grampp, and Schor, *J Biophys Biochem Cytol* 11: 549-557, 1961). He found no evidence of loss of RNA after a carefully controlled Carnoy's fixation. Also, it does not seem to me you, at any time, are offering evidence that you are not dealing entirely with a CCA terminal turnover, which would, of course, be expected to occur in the cytoplasm.

Woods: In regard to labeled nucleosides remaining in the tissue sections after freeze ethanol-substitution of fixation with 67% ethanol, I believe they are probably removed during the processing of the tissue prior to autoradiography. Nucleosides are quite soluble in ethanol and after a mild fixation, as we have done, they should easily be removed. In the column studies we don't recover nucleosides, probably because after phenol treatment and before analysis with the column the RNA extract is dialyzed and any nucleosides present would be washed away. However, in the autoradiographic studies, even if labeled nucleosides remain in the tissue and are not removed during our extraction step, these would not appear with tRNA because tRNA is extracted and determined by subtraction of the autoradiograph of the extracted section from the autoradiograph of the unextracted section. If the nucleosides were removed by the extraction step and not prior to tRNA extraction, then there would be a problem of distinguishing labeled precursor from labeled tRNA. The best evidence that this does not occur is seen in the observations illustrated in text-figure 3, where the curve for presumably total labeled tRNA for the whole cell extrapolates linearly to zero during all the isotope treatment periods. I don't believe the curve would be linear if labeled cytidine also was being extracted along with the tRNA. The smaller cytidine molecules might come out faster than the larger tRNA molecules making the curve nonlinear.

In regard to loss of RNA from sections of Carnoy-fixed tissue, I'm afraid I must disagree with Edström's findings. Our present autoradiographic data differ markedly from our earlier data, perhaps because different fixatives were used. In the earlier work the tissues were fixed in Carnoy's, and I believe that some of the tRNA must be removed by this fixative. Between 10 and 15% of the total RNA in our cells is tRNA. Perhaps the analytical methods used by Edström were not sensitive enough. I am not familiar with the details of his experiments.

Sirlin: Our fractions are treated in the cold for 30 minutes with 5% TCA, 6 seconds of 45% acetic acid, again 30 minutes of 5% TCA, then 30 minutes in buffer at pH 5.1, then about 15 minutes in distilled water at room temperature. When we look at these fractions, we still find uridine coming out of the precipitated RNA, even in the last fraction.

Woods: In regard to CCA terminal turnover, I believe that probably some H^3 -cytidine does become added to the end of the tRNA precursor molecule; however, the rest of the molecule must also become radioactive as new tRNA molecules are

synthesized. The number of terminal cytidine residues that might turn over is very small compared with the number of pyrimidine residues occurring in the rest of the molecule. I believe we are observing net synthesis of tRNA. I do not exclude the possibility that H^3 -cytidine is incorporated into the end of the molecule. In fact, I believe this does occur and is a good explanation for the rapid rise of label in the cytoplasm at the start of the experiment, as can be seen in text-figure 3. Here also we see a suggestion of a steady-state period occurring in the chromatin at about 45 minutes. This suggests that labeled tRNA is leaving the chromatin at a rate equal to its rate of synthesis in this structure. We would expect the cytoplasm, then, to show a time lag of about 45 minutes. Since this is not observed, I believe some terminal addition of H^3 -cytidine is taking place in the cytoplasm.

Comb: I would like to compliment Dr. Woods on this very nice method which is, in my opinion, very badly needed for interpreting autoradiographic data, and I am sure I speak for many other biochemists. In regard to the second low-molecular-weight-RNA peak you showed, we have changed our viewpoint on this. We believe from our recent studies that this is not a precursor of tRNA, and I will try to clarify this situation later. In *Escherichia coli*, it is bound to the 50S ribosome and looks very much like tRNA. What function it has, we do not know.

Woods: We haven't completed our experiments. We are doing them now, and maybe we will find something interesting in regard to this intermediate peak.

Feinendegen: Dr. Woods, was I correct in observing that in text-figure 3 the nucleolar evaluation stopped at 60 minutes and did not continue at 1 and 2 hours? Do you have any data showing the nucleolar activity in the chase experiment? Does this imply that you didn't see any?

Woods: No, we didn't make counts for the nucleolus in the chase experiment, but we did observe the slides. I am convinced that there is no labeled tRNA in the nucleolus, even in the turnover part of the experiment.

Waddington: Could I, as a nonbiochemist, ask a question? You have a reduction in the number of grains after treatment with a certain extraction procedure. The amount of reduction will depend on the proportion of tRNA to all the other RNA's left behind. It seems to me that you haven't given us any data about that. If tRNA is a small fraction of the total nucleolar RNA, even extracting the whole of it will make very little reduction in the grain counts. And, unless one has data on the proportion of tRNA to the total, can you interpret the amount of reduction you get?

Woods: I do not believe it is necessary to have data on the proportion of tRNA to total RNA for each compartment. What we are dealing with here is number of silver grains per unit volume of cellular structure (nucleolus, chromatin, cytoplasm) which is simply concentration of silver grains for each structure. By taking the difference between these grain concentrations before and after extraction, we obtain a value that represents grain concentration caused by only the tRNA in each compartment. These grain-concentration values can be taken to represent concentration of labeled tRNA in each compartment, and these are what we plot in our curves with respect to time. Now, I will agree that in some cases where labeled nontransfer RNA occurs in high concentration, difficulties are encountered in obtaining accurate data for labeled tRNA. We have attempted to overcome these difficulties by counting large numbers of cells.

Pelling: The technique you used for 4S RNA should also be considered for RNA fractions of higher molecular weight. At present, we are examining how treatment with certain fixatives affects the density gradient patterns of radioactive RNA. So far we have found remarkable differences between different fixatives.

Birnstiel: You have shown very nicely that you can extract tRNA. You obtain a yield of up to 87% and this RNA has an activity as tRNA. But the question is, where is the other 13%, and could it be nucleolus-bound?

Woods: I don't know. Certainly we would not see it autoradiographically; we would not see the part that is unremoved. It may well be it is in the nucleolus. Further study may answer that.

Birnstiel: Have you extended your Sephadex studies to the labeled RNA to see whether the labeled RNA you removed is, in fact, coincident with the tRNA peak?

Woods: No, we have not done that.

Perry: Have you tried H^3 -guanosine as a precursor? This would definitely, I think, solve the problem of the amount of terminal addition because with guanosine you would not get any terminal incorporation or any significant conversion. Have you tried this or do you intend to try it?

Woods: No, but it sounds like a good suggestion. It should be done.

Nucleolar Enzymes of Isolated Rat Liver Nucleoli^{1, 2}

GÜNTHER SIEBERT,³ *Department of Pharmacology,
Baylor University College of Medicine, Houston, Texas*

SUMMARY

By means of the mass isolation procedures of Busch and co-workers, nucleoli were prepared from rat liver nuclei isolated in 2.2 M sucrose. Assays for glutamate dehydrogenase, adenylate kinase, catalase, acid phosphatase, 5'-nucleotidase, glucose-6-phosphatase, lactate dehydrogenase, and pyruvate kinase as marker enzymes for cytoplasmic contamination revealed that the isolated nucleoli were in a high state of purity. The intranuclear distribution patterns and the specific activities of nuclear enzymes were studied in different nuclear sub-fractions. RNA polymerase, ribonuclease, and, to a lesser extent, NAD pyrophosphorylase and adenosinetriphosphatase A are localized in the nucleolus. Nuclear acid and alkaline deoxyribonuclease and adenosinetriphosphatase B were mainly found in the non-nucleolar fractions. Sonication, Triton X-100, and *p*-chloro-

mercuribenzoate produced an increase of ribonuclease activity in nuclear and nucleolar preparations. Such nuclear fractions from normal rats and from rats treated with thioacetamide or actinomycin D, or both, responded differently to treatment by sonication, Triton X-100, or *p*-chloromercuribenzoate. Therefore, there may be more than one mechanism of latency of nuclear and nucleolar ribonucleases which would seem to depend on the changes in RNA metabolism brought about by treatment with thioacetamide or actinomycin D, or both. The substrate specificity of ribonuclease, adenosinetriphosphatase A, and adenosinetriphosphatase B of nuclear and nucleolar origin are different and point to qualitative differences between these nuclear and nucleolar enzymes.—*Nat Cancer Inst Monogr* 23: 285–293, 1966.

ONLY a few data exist on enzymes in nucleoli. For example, the following enzymes have been detected in isolated nucleoli from various sources: alanyl-glycine dipeptidase (1), alkaline and acid phosphatase (1), adenosine deaminase (2), guanase (2), ribonucleases (3, 4), nucleoside phos-

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported in part by grants from the American Cancer Society, Inc., the Jane Coffin Childs fund, the National Science Foundation, and U.S. Public Health Service grant CA-08182 from the National Cancer Institute.

³ Present address: Department of Physiological Chemistry, Johannes Gutenberg University, Mainz, Germany, P.O.B. 606.

phorylase (2), DPN pyrophosphorylase (2), RNA polymerase (5-8), RNA methylase (9, 10), and amino acid-activating enzymes (11). Since mass isolation procedures for nucleoli from rat liver are now available (12), a systematic study of nucleolar enzymes was undertaken (13) with nucleoli isolated from rat liver by the sonication procedure of Busch and co-workers.

Although metabolic experiments, mostly by isotope methods, yield some clues as to enzymic activities in nucleoli [for a review *see* (14, 15)], systematic investigations of nucleolar enzymes seem urgent for an elucidation of detailed molecular events during nucleolar activity.

Recent progress in studies of the enzymology of the nucleus (16) has furnished some experimental designs which seem applicable to enzymology of the nucleolus: 1) The purity of isolated nucleoli, *i.e.*, the absence of cytoplasmic contaminants, may be assessed by the use of marker enzymes as well as by morphological and analytical observations. 2) The well-known technique of studying the intracellular distribution of enzymes, by determining total and specific activities in all subfractions of a homogenate, may be applied to whole nuclei and their subfractions, thus yielding the complete intranuclear distribution patterns of nuclear enzymes.

MATERIALS AND METHODS

Nucleoli were isolated from rat liver nuclei by the sonication procedure of Busch and co-workers (12). The assays for enzymes were performed by standard procedures (13).

RESULTS

Purity of Isolated Nucleoli

Results with typical marker enzymes are given in table 1. In every case, the extent of nuclear contamination by cytoplasmic material was only a small percentage of the activity of these marker enzymes in the whole homogenate. From this small amount of contamination in nuclei, only a fraction is carried down into the nucleolar preparations, as may be seen from the specific activities of the marker enzymes, and from the usually very small percentage recovered in the nucleoli. For instance, catalase as an indicator of microbodies was found in nucleoli with $7 \times 10^{-4}\%$ of the activity of the whole liver homogenate. Therefore, enzymic studies on isolated nucleoli are not seriously hampered by cytoplasmic contaminants. Also, as shown previously (12, 14, 15), the high degree of purity of isolated nucleoli is demonstrated by these assays.

TABLE 1.—Studies with marker enzymes in isolated nucleoli of rat liver

Enzyme	Nuclei		Nucleoli	
	Specific activity*	Percent†	Specific activity*	Percent†
Glutamic dehydrogenase.....	5. 4	100	2. 5	5
Adenylate kinase.....	60	100	24	3
Catalase.....	3500	100	1800	4
Acid phosphatase.....	5. 3	100	2. 3	30
5'-Nucleotidase.....	6. 2	100	2. 5	23
Glucose-6-phosphatase.....	33	100	0	0
Lactate dehydrogenase.....	220	100	240	8
Pyruvate kinase‡.....	20	100	40	9

*m μ mol/mg protein \times minute⁻¹.

†Percent of nuclear activity recovered in nucleoli.

‡Partial inactivation by sonication.

Intranuclear Distribution of Enzymes

From table 2 it may be seen that the specific activity of RNA polymerase is more than 20 times higher in nucleoli than in nuclei. Since nucleolar mass and nucleolar protein in normal liver contribute about 6% to nuclear mass and nuclear protein (12), one must conclude that RNA polymerase is exclusively localized in the nucleolus. Ribonuclease, and to a lesser extent DPN pyrophosphorylase and adenosinetriphosphatase A, is preferentially localized in the nucleolus, whereas the two deoxyribonucleases are rather uniformly distributed within the nucleus. Adenosinetriphosphatase B, on the other hand, is apparently of extranucleolar localization. Thus, these data strongly support the concept (16) of intranuclear compartmentalization of enzymes.

TABLE 2.—Intranuclear distribution of nuclear enzymes in rat liver

Enzyme	Specific activity	
	Nuclei	Nucleoli
RNA polymerase..... (cpm/mg protein in 20 minutes)	140	3300
Ribonuclease..... (Δ OD/mg protein in 30 minutes)	0. 82	5. 0
DPN pyrophosphorylase..... (m μ mol/mg protein \times minute ⁻¹)	7. 3	22
Adenosinetriphosphatase A..... (m μ mol/mg protein \times minute ⁻¹)	14	31
Acid deoxyribonuclease..... (Δ OD/mg protein in 30 minutes)	6. 1	12
Alkaline deoxyribonuclease..... (Δ OD/mg protein in 30 minutes)	7. 8	12
Adenosinetriphosphatase B..... (m μ mol/mg protein \times minute ⁻¹)	21	6

Besides these quantitative differences, there are marked qualitative differences between nuclear and nucleolar enzymes as may be seen from specificity studies on ribonuclease and the two adenosinetriphosphatases. If one relates cleavage rates of ATP with those of any other nucleoside triphosphate, ratios of 0.28 and 5.0 are found for UTP with the enzyme A, and for GTP with the enzyme B, respectively (table 3). In the light of detailed studies on the specificity of nuclear adenosinetriphosphatases (16), these results indicate profound differences between the respective nuclear and nucleolar enzymes. Similar findings were obtained with ribonuclease, where significantly higher rates of the nuclear than of the nucleolar enzyme with nuclear 18S RNA and polyadenylate were observed, whereas other substrates were not cleaved at markedly different rates (table 4). Such studies, still in the preliminary stage, suggest the individuality of nuclear and nucleolar ribonuclease.

TABLE 3.—Specificity of adenosinetriphosphatases A and B in nuclei and nucleoli of rat liver

Substrate	Enzyme A	Enzyme B
	$\frac{\text{ATP}}{\text{XTP}}$ in nuclei*	
	$\frac{\text{ATP}}{\text{XTP}}$ in nucleoli	
GTP.....	0. 71	5. 0
CTP.....	1. 8	1. 2
UTP.....	0. 28	1. 5
dATP.....	0. 65	2. 0
	$\frac{\text{dATP}}{\text{dXTP}}$ in nuclei†	
	$\frac{\text{dATP}}{\text{dXTP}}$ in nucleoli	
dGTP.....	0. 64	0. 34
dCTP.....	1. 5	0. 86
TTP.....	2. 0	0. 25

*X=G, C, or U.
†dX=dG, dC, or T.

Activity and Latency of Ribonuclease

Experimental conditions known to alter the cellular metabolism of RNA lead to considerable change in the activity of ribonuclease (table 5). The increase of total activity caused by treatment with thioacetamide is twice as high in the nucleolus as in the nucleus; yet, the increase of nucleolar mass after treatment with thioacetamide apparently exceeds the increase of nucleolar ribonuclease since the ratios of specific activities between

TABLE 4.—Specificity of ribonuclease in nuclei and nucleoli in rat liver

Substrate	Nuclei after sonication*	Nucleoli*	$\frac{\text{Nuclei}^\dagger}{\text{Nucleoli}}$
Ribosomal RNA.....	100	100	0.92
Nuclear RNA, 18S.....	79	41	1.73
Nuclear RNA, 28S.....	87	92	0.87
Nuclear RNA, 35S.....	108	88	1.15
Nuclear RNA, 45S.....	161	147	1.02
Polyadenylate.....	33	16	1.94
Polyuridyate.....	0	0	—

*Relative rates, with ribosomal RNA = 100%.

†Ratio of actual rates.

TABLE 5.—Activity and latency of ribonuclease in nuclei and nucleoli of rat liver

	Pretreatment of rats			
	Control	Thioacetamide	Actinomycin D	Thioacetamide plus actinomycin D
Total activity*				
Nuclei.....	3.3	6.5	0.94	4.5
Nucleoli.....	1.3	5.5	0.04	3.5
Nucleoli/nuclei.....	0.40	0.85	0.04	0.78
Specific activity†				
Nuclei.....	0.82	1.5	0.90	1.5
Nucleoli.....	5.0	3.1	0.80	4.4
Nucleoli/nuclei.....	6.1	2.1	0.89	2.9
% "activation" by sonication‡....	+51	+142	+210	+95
0.1% Triton X-100.....	+85	+25	0	0
3×10^{-4} M <i>p</i> -CMB.....	+49	0	+118	0
2×10^{-3} M mercaptoethanol.....	+23	0	+93	0

* Δ OD/30 minutes \times g liver⁻¹.† Δ OD/30 minutes \times mg protein⁻¹.‡Figures for Triton X-100, *p*-CMB (*p*-chloromercuribenzoate) and mercaptoethanol refer to nuclei, nuclei after sonication, and nucleoli.

nucleolar and nuclear ribonuclease declined from 6.1 to 2.1. Quite in contrast are results after pretreatment with actinomycin D where the decrease of activity is much more pronounced with the nucleolar than with the nuclear ribonuclease. These data seem to suggest that the intensity of RNA metabolism in the nucleus would be correlated with the activity of nuclear and nucleolar ribonucleases. However, one might wish to remain open-minded as to whether the inhibition of RNA synthesis by actinomycin D causes the decreased ribonuclease activity or whether actinomycin D acts in a direct and RNA-independent manner on ribonuclease activity.

Under several conditions, a latency of ribonuclease is revealed, *e.g.*, sonication enhances the activity of whole nuclei considerably, the extent

of "activation" depending on the pretreatment of rats (table 5). Triton X-100 is capable of increasing the activity in nuclear fractions before, as well as after, sonication. It would seem, therefore, that the effect of non-ionic detergents like Triton X-100 indicates a mechanism of latency which should be different from that detected after sonication. Finally, *p*-chloro-mercuribenzoate (*p*-CMB), which is known to bind the ribonuclease inhibitor (17), demonstrates another means of activity regulation of ribonuclease that should be independent of those revealed by sonication and detergents. It is noteworthy that the extent and type of latency varies in every experimental group, *e.g.*, nuclear and nucleolar ribonucleases from normal rats respond to all "activating" means, whereas after treatment with thioacetamide, *p*-CMB no longer produces an increase in activity but sonication is much more effective. Actinomycin D seems to cause a loss of response to detergents but an appearance of activation by SH-compounds and a much higher effect of *p*-CMB, whereas after treatment with thioacetamide plus actinomycin D only the activation by sonication is retained.

At least a part of the latency may be caused by a binding of this enzyme to structural components of the nucleus or nucleolus, or both. In addition, ribonuclease cannot be extracted by conventional means from either nuclei or nucleoli. This structural binding of ribonuclease would therefore be one of the factors contributing to the very intricate and sensitive system of activity regulation revealed by this study of latency. However, structural binding has some general effects on enzyme kinetics in that the formation of enzyme-substrate complexes has to occur between an immobile enzyme and a moving substrate molecule, instead of both partners moving around which is the usual and much simpler case. Also, gel-sol transitions of nuclear components, as perhaps best demonstrated by chromosome formation during mitosis or by the puffing phenomenon, may well affect the structural binding of enzymes, with possible consequences as to the actual ribonuclease activity in different states of nuclear and/or nucleolar activity.

Another part of latency of ribonuclease should be ascribed to the ribonuclease inhibitor of rat liver. Thioacetamide and actinomycin D seem to act, as seen by the activation by *p*-CMB, in a very opposite manner on the activity of this inhibitor in nuclei and nucleoli.

DISCUSSION

The nucleolus is one of the cell's centers of RNA metabolism, probably representing for the whole cell the same importance in ribosomal RNA metabolism as, for example, the mitochondria in electron transport. The experiments reported here demonstrated that, despite homogenization in aqueous media, sonication, and other steps taken, nucleoli retain a high degree of structural organization after their isolation.

The study of nucleolar enzymes concerned with RNA metabolism is complicated by problems concerning their latency, but the question certainly does not lose its challenge to investigators (18-20). The nucleolar role in RNA metabolism is one of the main events in gene activity; its deeper understanding in molecular terms may depend on progress in the knowledge of activity regulation of nucleolar enzymes.

RESUMEN

Mediante los procedimientos de aislamiento en masa de Busch y colaboradores se prepararon nucleolos a partir de núcleos aislados de hígado de rata en sacarosa 2.2 M. Los ensayos con glutamato dehidrogenasa, adenilato quinasa, catalasa, fosfatasa ácida, 5'-nucleotidasa, glucosa-6-fosfatasa, lactato dehidrogenasa y piruvato quinasa como enzimas marcadoras para contaminación citoplásmica revelaron que los nucleolos aislados estaban en un elevado estado de pureza. Se estudiaron las enzimas nucleares en su patrón de distribución intranuclear y sus actividades específicas en diferentes subfracciones nucleares. La ARN polimerasa, ribonucleasa y, en menor extensión, la NAD pirofosforilasa y ATPasa A se localizan en el nucleolo. El ácido nuclear y la deoxirribonucleasa alcalina y la ATPasa B se hallaron principalmente en las fracciones no nucleolares. Los ultrasonidos, el Triton X-100 así como el p-cloromercuriobenzoato produjeron un aumento de la actividad de la ribonucleasa en las preparaciones nucleares y nucleolares. Dichas fracciones nucleolares de ratas normales y de ratas tratadas con tioacetamida y/o actinomicina D respondieron en forma diferentes al tratamiento con ultrasonidos, el Triton X-100 o el p-cloromercuriobenzoato. Además, puede haber más de un mecanismo de latencia de las ribonucleasas nucleares y nucleolares que parece depender de los cambios en el metabolismo del ARN efectuados por la tioacetamida y/o la actinomicina D. Las especificidades del sustrato de la ribonucleasa, ATPasa A, y ATPasa B de origen nuclear y nucleolar son diferentes y señalan diferencias cualitativas entre estas enzimas nucleares y nucleolares.

REFERENCES

- (1) VINCENT, W. S.: The isolation and chemical properties of the nucleoli of starfish oocytes. *Proc Nat Acad Sci USA* 38: 139-145, 1952.
- (2) BALTUS, E.: Observations sur le rôle biochimique du nucléole. *Biochim Biophys Acta* 15: 263-267, 1954.
- (3) ———: Étude de la ribonucléase des nucléoles isolés à partir d'oocytes d'asteries (*Asterias rubens*). *Biochim Biophys Acta* 55: 82-87, 1962.
- (4) VILLALOBOS, J. G., JR., STEELE, W. J., and BUSCH, H.: Ribonuclease activity of isolated nucleoli of livers of thioacetamide-treated rats. *Biochim Biophys Acta* 103: 195-200, 1965.
- (5) TSUKADA, K., and LIEBERMAN, I.: Synthesis of ribonucleic acid by liver nuclear and nucleolar preparations after partial hepatectomy. *J Biol Chem* 239: 2952-2956, 1964.
- (6) LERMAN, M. I., MANTIEVA, V. L., and GEORGIEV, G. P.: Biosynthesis of ribosomal RNA in the nucleolus. (The nucleonemal apparatus of the cell.) *Biochimii (Moscow)* 29: 518-528, 1964.
- (7) RO, T. S., MURAMATSU, M., and BUSCH, H.: Labeling of RNA of isolated nucleoli with UTP-¹⁴C. *Biochem Biophys Res Commun* 14: 149-155, 1964.

- (8) Ro, T. S., and BUSCH, H.: *In vitro* labeling of RNA in isolated nucleoli of the Walker tumor and liver. *Cancer Res* 24: 1630-1633, 1964.
- (9) BIRNSTIEL, M. L., FLEISSNER, E., and BOREK, E.: Nucleolus: a center of RNA methylation. *Science* 142: 1577-1580, 1963.
- (10) COMB, D. G., and KATZ, S.: Studies on the biosynthesis and methylation of transfer RNA. *J Molec Biol* 8: 790-800, 1963.
- (11) BRANDT, E. E., and FINAMORE, F. J.: Protein synthesis in frog eggs. II. Amino acid activation and incorporation by isolated nucleoli. *Biochim Biophys Acta* 68: 618-624, 1963.
- (12) MURAMATSU, M., SMETANA, K., and BUSCH, H.: Quantitative aspects of isolation of nucleoli of the Walker carcinosarcoma and liver of the rat. *Cancer Res* 23: 510-518, 1963.
- (13) SIEBERT, G., VILLALOBOS, J. G., JR., Ro, T. S., STEELE, W. J., LINDENMAYER, G., ADAMS, H., and BUSCH, H.: Enzymatic studies on isolated nucleoli of rat liver. *J Biol Chem* 241: 71-78, 1966.
- (14) VINCENT, W. S.: Structure and chemistry of nucleoli. *Int Rev Cytol* 4: 269-298, 1954.
- (15) BUSCH, H., BYVOET, P., and SMETANA, K.: The nucleolus of the cancer cell. A review. *Cancer Res* 23: 313-339, 1963.
- (16) SIEBERT, G., and HUMPHREY, G. B.: Enzymology of the nucleus. *Advances Enzym* 27: 239-288, 1965.
- (17) ROTH, J. S.: Ribonuclease. VII. Partial purification and characterization of a ribonuclease inhibitor in rat liver supernatant fraction. *J Biol Chem* 231: 1085-1095, 1958.
- (18) ———: Ribonuclease. VIII. Studies on the inactive ribonuclease in the supernatant fraction of rat liver. *J Biol Chem* 231: 1097-1105, 1958.
- (19) SHORTMAN, K.: Studies on cellular inhibitors of ribonuclease. I. The assay of the ribonuclease-inhibitor system, and the purification of the inhibitor from rat liver. *Biochim Biophys Acta* 51: 37-49, 1961.
- (20) ———: Studies on cellular inhibitors of ribonuclease. II. Some properties of the inhibitor from rat liver. *Biochim Biophys Acta* 55: 88-96, 1962.

DISCUSSION

Mandel: I agree entirely with you that it is difficult to say anything about enzymatic activity in nuclei when the nuclei are isolated in aqueous solvents. We found the presence of a soluble RNA polymerase without traces of DNA in nuclei which nobody else could find because they lost it during the isolation (Ramuz, Doly, Mandel, and Chambon, *Biochem Biophys Res Commun* 19: 114-120, 1965). What is lost during the preparation of nuclei or nucleoli in aqueous solvents is always a problem. How do you relate your specific activity to protein? As you said, 95% of the proteins in the nucleoli as well as in the nucleus are nonenzymatic proteins.

Siebert: We determined the total soluble proteins, as is usually done with crude tissue extracts. What we could do, although it's difficult, would be to subtract from total protein the histones, the residual proteins, or whichever fraction we thought might be nonenzymatic. But we are not sure how many of nucleolar proteins are enzymes, maybe 0.5, maybe 5, perhaps 15%. As we cannot distinguish between the total group of enzymic and the total group of nonenzymic proteins, I do not think we can find a better baseline now.

Cohen: I would like to make a few comments and I'll comment first on the question Dr. Mandel asked. It is highly desirable to express enzyme activity as *specific activity* (units of enzyme activity/mg protein). The fact that the enzyme represents only a small part of the total protein of the extract is a situation which occurs in the assay of any tissue extract. The advantage of expressing the enzyme activity

as *specific activity* is that it provides an index of the amount of the total protein which is represented by that particular enzyme, and an increase or decrease of *specific activity* provides information whether the enzyme activity is increasing or decreasing independently of the changes in total protein. The fact that a certain amount of enzyme is "bound" (and presumably inactive) raises a question. Did you assay the nucleolar extracts in the presence of added nuclear extract? Does the nuclear extract have any effect on the enzyme activity of the nucleolar extract? In other words, are the enzyme activities of the extract of the nucleus and that of the nucleolus additive when mixed? Finally, I would like to say that while I consider Dr. Siebert's contribution very important, the significance of his findings will depend on whether the levels of the enzymes assayed are present in the cell in rate-limiting concentrations. If an enzyme is present in excess of its rate-limiting concentration in all instances, then it is difficult to assign metabolic significance to a small increase or decrease.

Siebert: As to the first question, we did a complete recovery balance of activities in different subfractions, and when recombining single fractions, we never got an indication of an over- or under-additive behavior. As to the second point, I think that most intracellular enzymes do not determine rates, but that rates are determined in many cases by substrate concentrations. And I also refer to Dr. Mandel's remark. What we are going to try is nonaqueous isolation of nucleoli under conditions which will permit us to determine actual substrate concentrations in these particles where we also know the enzymic activities. Until we can obtain data on substrate concentrations, I would agree completely with you that a change in enzyme activity does not necessarily reflect a change in over-all metabolism. However, at the very moment when one enzyme competes with another for a common substrate, a change in the activity of one enzyme would shift the relative rate with which a substrate goes to one or the other enzyme.

Busch: I just would like to make two comments. One is that studies in enzymology, such as those carried out in our group by Ro (Ro and Busch, *Cancer Res* 24: 1630-1633, 1964) and Villalobos (Villalobos, Steele, and Busch, *Biochim Biophys Acta* 103: 195-200, 1965), and much more extensively by Dr. Siebert, provide us with a clearer indication of the need of improvement of technology for mass isolation of nucleoli as well as the specific problems associated with aqueous and nonaqueous nuclei. We are really just at a beginning in this field. The second point, however, is that only by the isolation of individual molecular species of enzymes will one be able to distinguish whether there are specific nucleolar enzymes as opposed to the extranucleolar nuclear enzymes.

Perry: If I understood the data you presented concerning thioacetamide-treated animals, the specific activity of the nucleolar ribonuclease is higher than that of the nuclear ribonuclease. And yet, in this case, the nucleolar RNA is much higher than normal. How do you explain that an increase of a degradative enzyme could be correlated with an increase in the RNA itself? I find this a rather surprising result. Had you gotten the opposite result, it would have been much easier to understand.

Siebert: The common presence of both proteins and proteolytic enzymes in every living cell is a similar situation. I have no data to support this, but structural binding of ribonuclease would mean limited availability of substrate. I do not think we can say more than that we must accept the fact that increased RNA synthesis and increase in nucleolar mass and volume are accompanied by an increase in total ribonuclease activity. To what extent this total activity is suppressed by regulating factors remains open.

THE NUCLEOLUS IN THE CELL CYCLE AND DURING DEVELOPMENT

One of the earliest observations on the nucleolus which led to speculations on its function was the fact that, although it is very conspicuous in oogenesis, it is absent during cleavage and early embryogenesis. Likewise, it was noted that the nucleolus was obvious during interphase of the normal cell cycle but disappeared during mitosis. Such periods of nucleolar disappearance and the predictable reappearance have made the cell cycle, oogenesis and early development, favored material for the study of nucleolar metabolism with a wide variety of techniques, particularly autoradiography. The papers which follow include the results of biochemical as well as autoradiographic studies, and also introduce the effects of certain antimetabolites and inhibitors on nucleolar chemistry and function.

This portion of the Symposium was chaired by Dr. M. E. Drets, Dr. J. I. Valencia, Dr. R. C. von Borstel, and Dr. G. Favelukes.

The Nucleolus and Synthesis of Ribosomal RNA During Oogenesis and Embryogenesis of *Xenopus laevis*¹

DONALD D. BROWN,² *Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland 21210*

SUMMARY

The following observations of oocytes and developing embryos strongly support the relationship between the presence and size of nucleoli with the presence and rate of synthesis of ribosomal RNA.

1) The number of nucleoli in oocytes does not correspond to the expected ploidy. The unusually large number or size of nucleoli in oocytes correlates with the extraordinarily high synthetic rate of rRNA which is characteristic of growing oocytes. 2) When the oocyte begins meiosis, nucleoli disappear and the synthesis of rRNA ceases. 3) Early development of many embryos is characterized by the absence of both visible nucleoli and synthesis of rRNA. 4) For the amphibian, the onset of synthesis of rRNA correlates exactly with the first appearance of visible nucleoli at gastrulation. As development proceeds, nucleoli increase in size and the rate of rRNA synthesis increases. 5) An anucleolate mutant of *Xenopus laevis* has no definitive nucleoli and does not synthesize rRNA. 6) By nuclear

transplantation it has been shown that the cytoplasm of unfertilized eggs and cleaving embryos can inhibit both the synthesis of rRNA and nucleolar appearance. 7) Whereas the synthesis of rRNA correlates precisely with the presence and size of nucleoli, the synthesis of DNA-like RNA and 4S RNA is completely unrelated to nucleolar size or presence. The high rate of synthesis and remarkable stability of ribosomes in oocytes result in large numbers of ribosomes in the unfertilized egg relative to the other two classes of RNA (DNA-like RNA and 4S RNA). These ribosomes support protein synthesis throughout amphibian development up to the early swimming stage. During early development the synthesis of rRNA and 4S RNA is so intense relative to ribosome synthesis that their relative deficiency in the egg is corrected. In this way the developing embryo increases its capacity to synthesize protein.—Nat Cancer Inst Monogr 23: 297–309, 1966.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² I am indebted to Miss Elizabeth Littna for her assistance with all the experiments performed in this laboratory, to my colleagues Mr. R. Hallberg, Mrs. M. Schwartz, and Dr. C. Weber for their critical comments on the manuscript, and finally to Dr. A. A. Neyfakh for providing several references for table 3.

THIS PAPER describes the changing pattern of nucleolar number and size in growing oocytes and developing embryos and its relation to the synthesis of ribosomal RNA. The presence of nucleoli, as well as their number and size in each cell, gives specific information on a molecular level. Generally the number of nucleoli corresponds to the ploidy of a cell (and the degree of heterozygosity in animals with mutations in the nucleolar organizer region). The presence of nucleoli indicates the cell is synthesizing ribosomal RNA (rRNA).³ Finally, nucleolar size correlates with the *rate* of ribosomal RNA synthesis. Therefore, the nucleolus is a visible organelle whose presence assures the presence of a defined set of genes; these genes define the nucleotide sequence of ribosomal RNA.

NUCLEOLI AND THE SYNTHESIS OF RIBOSOMAL RNA DURING OOGENESIS

The oocyte is a cell in which the number of nucleoli does not define its ploidy (1). Many oocytes, although 4N in chromosome complement, have a single nucleolus which enlarges to an extraordinary size during early oogenesis before yolk accumulation begins (1, 2). In contrast, oocytes of amphibia and fish have thousands of nucleoli per nucleus (3). These multiple nucleoli are comparable to somatic nucleoli in their ultrastructure (4, 5). The following evidence suggests that they are the sites where rRNA is synthesized:

- 1) Microanalyses by Edström and Gall (6) have demonstrated a high guanylic-ctidylic acid (G-C) content for nucleolar RNA, which is similar to cytoplasmic (ribosomal) RNA but differs from chromosome-associated RNA (low % G-C).
- 2) Kinetics of labeling with H³-nucleosides studied by radioautography shows that RNA of nucleoli is synthesized as rapidly as chromosomal RNA (5, 7). This labeling is inhibited by actinomycin D (8).
- 3) Nucleoli of larger oocytes continue to incorporate radioactive precursors into RNA even after the lampbrush chromosomes have contracted and have stopped synthesizing RNA (7). During this late period of oogenesis, it has also been shown that over 95% of the RNA synthesized is ribosomal RNA (9, 10).
- 4) Radioautographic studies have failed to demonstrate any substantial contribution of preformed RNA to amphibian oocytes from maternal sources. These data support the view that the RNA of an oocyte is a product of its own nucleus.

Oogenesis results in the formation of a large single cell, the egg, which has as many ribosomes per wet weight of tissue as a multicellular tissue such as liver. Table 1 summarizes the rRNA and DNA contents of liver and unfertilized eggs of *Xenopus laevis*, the South African "clawed toad." The egg has approximately the same content of ribosomes per wet weight as liver, but only about 5% of the 4S RNA (11). Furthermore, it has only 0.1% as much DNA as liver and this DNA is homologous to only a fraction of liver DNA (12). The nuclear value (4N) is calculated from the known

³ Abbreviations are: rRNA—ribosomal RNA, dRNA—DNA-like RNA, rDNA—DNA complementary in base sequence to ribosomal RNA and presumably the template for its synthesis, % G-C—mole % guanylic-ctidylic acid.

diploid content of erythrocytes which is 6 μg (12). Therefore, the same wet weight of liver should have about 200,000 times more nuclear DNA than an unfertilized egg. By the time the embryo has completed its development, the relative amounts of rRNA, 4S RNA, and DNA per wet weight are comparable to adult liver (table 1).

TABLE 1.—The DNA and RNA content of eggs, tadpoles, and liver of *Xenopus laevis*

	$\mu\text{g}/\text{mg}$ wet weight		
	Egg	Swimming* tadpole	Liver
Total DNA†.....	3	2000	2000
"Nuclear" DNA†.....	0.012	2000	2000
rRNA.....	4000	8000	6000
Low molecular weight RNA‡.....	80	600	900

*These embryos have just completed development and are ready to feed [Nieuwkoop-Faber (15) stage 45].

†One egg weighs approximately 1 mg. Values for total and "nuclear" egg DNA are taken from Dawid (12). "Nuclear" DNA is the expected value for a nucleus with a 4N chromosome complement. The unfertilized egg nucleus is still diploid having only completed one meiotic division. The polar body containing the other two pairs of chromosomes has not separated from the egg and is isolated with it when the egg is homogenized.

‡This RNA fraction includes both 4S and 5S RNA (13) and was purified by passing RNA preparations from eggs, embryos, or liver through Sephadex G 100 columns (11). The RNA which is retarded by the columns was purified further by fractionation on methylated serum albumin columns.

The remarkable rRNA content of eggs (per DNA complement) could be a result of the stability of ribosomes accumulated over a long period of oogenesis, while ribosomes of liver are being formed at a much greater rate but are being degraded more rapidly. To test this hypothesis, female *X. laevis* were induced to ovulate and then injected with $^{32}\text{PO}_4$ intraperitoneally 24 hours later. Three days after injection, the animals were killed and the ribosomal RNA was purified from liver, ovarian tissue, and oocytes of different sizes. The specific activities of the rRNA are summarized in table 2. Similar relative values for oocytes and liver were obtained in several experiments at periods after ^{32}P injection ranging from 48 hours to 7 days. These values do not give absolute rates of synthesis, because differences in the specific activities of the precursor pools in different tissues have not been measured. Nevertheless, growing oocytes synthesize rRNA at rates comparable to multicellular liver, despite the fact that they contain only 0.1% as much total DNA per wet weight (see table 1). Furthermore, ribosomes in oocytes are extremely stable (9, 14) and may in fact be conserved throughout oogenesis. In oocytes of *X. laevis*, ribosomes labeled more than 1 year previously are still radioactive (9).

There are two explanations to account for the remarkable synthetic rate of rRNA in the oocyte. One hypothesis suggests that the DNA template for rRNA (rDNA) of a somatic cell is never the limiting reactant

TABLE 2.—Specific activity of 28S ribosomal RNA of different tissues 64 hours after injection of ³²PO₄

Tissue*	cpm/ μ g RNA
Liver.....	12
Ovarian tissue.....	3
Small oocytes.....	7
Large oocytes.....	2

*One day after ovulation a female *X. laevis* was injected intraperitoneally with 1 mc of neutral sodium phosphate (³²P). The animal was left at room temperature for 64 hours and then killed. Small (less than 0.2 mm) and large (0.8 to 1.0 mm) oocytes were collected with a hairloop, and the RNA was extracted by the cold phenol-detergent method (18). Ovarian tissue was freed from most of the largest oocytes by gently pulverizing it with a pestle and washing it thoroughly. It was homogenized and the RNA extracted by the same procedure.

in the synthesis of rRNA. Therefore in oocytes the limiting component would have been supplemented so that the over-all rate of rRNA synthesis *per complement of ribosomal DNA* is greatly increased. Alternatively, it can be argued that each nucleolus contains extra copies of ribosomal DNA (templates for rRNA) so that this single cell can support greatly augmented rates of rRNA synthesis. The rate of rRNA synthesis per complement of rDNA is not fixed but is regulated in the most subtle fashion (16). The entire period of early development affords good evidence for this view, since synthesis of rRNA appears to be entirely *stage dependent* (16, 17).

However, to test the second hypothesis, *X. laevis* DNA from liver, erythrocytes, early and late embryos, and swimming tadpoles has been analyzed for the percentage of their genome which is complementary to purified ribosomal RNA. In all cases, about 0.1 % of the total DNA can be hybridized with rRNA. Therefore, great variations in the rates of rRNA synthesis presumably occur in cells that appear to have identical amounts of DNA complementary to rRNA (14). This hybridization test has not yet been carried out with egg DNA. The experiment is complicated by the large amount of egg DNA which is not nuclear but rather cytoplasmic (? mitochondrial) in character (12). This DNA represents multiple copies that are homologous with only a small portion of the frog genome (12). The dilemma can be summarized as follows. A 4N nucleus should contain 12 μ g of DNA; the frog egg contains 3000 μ g of DNA. Most of the excess copies in egg DNA cannot be rDNA (61% G-C) because its over-all base composition (40% G-C) is only slightly different from the nuclear DNA (42% G-C) (12). Even if we assume, for example, that the nuclear sites for rDNA are present in 2000 excess copies, this would represent about 24 μ g of rDNA per egg—only 0.8% of the total egg DNA.

RNA SYNTHESIS IN MATURE OOCYTES AND DURING MEIOSIS

The unusually large single nucleolus (or numerous nucleoli in the case of amphibian oocytes) remains intact when the oocyte has reached

maturity. During the first meiotic reduction division, the large nucleus (germinal vesicle) of the oocyte breaks down and the nucleolus (or nucleoli) disappears (1). The fate of these nucleoli is unknown. In amphibian oocytes, concomitant with the disappearance of nucleoli and the initiation of meiosis, there is a change in the type of high molecular weight RNA which is synthesized. Before meiosis, rRNA is the predominant class of RNA being synthesized while during meiosis predominantly heterogeneous dRNA is formed (18). In the amphibian embryo, this pattern of synthesis persists throughout meiosis and cleavage. Nucleoli reappear in cells of the embryo at the onset of gastrulation when the synthesis of rRNA begins (16).

ABSENCE OF NUCLEOLI AND RIBOSOMAL RNA SYNTHESIS IN A MUTANT STRAIN OF *X. LAEVIS*

The role of nucleoli in the synthesis of rRNA has been greatly advanced by studies with a mutant of *X. laevis* which has an altered number of nucleoli. Diploid cells of wild-type *X. laevis* have two nucleoli, but heterozygotes have only one nucleolus in each diploid cell while homozygous mutants are anucleolate. A mating of two heterozygotes yields progeny of the three genotypes in ratios of 1:2:1 [anucleolate (0-*nu*) : heterozygote (1-*nu*) : wild type (2-*nu*) (19,20)]. The anucleolate mutants die at the early swimming stage having synthesized no detectable rRNA or rRNA precursor molecules during the entire period of embryogenesis (21).

The recent studies of Wallace and Birnsteil (22) showed that this mutation is a deletion of the chromosomal region which is complementary to ribosomal RNA.

The mutation appears to be specific for ribosomal RNA and its precursors, since anucleolate embryos synthesize 4S RNA and higher molecular weight DNA-like RNA (21). This supports other evidence reported here that the synthesis of rRNA, the presence of a nucleolus, and a nucleolar organizer are neither correlated with, nor required for, the synthesis of dRNA or 4S RNA.

APPEARANCE OF THE NUCLEOLUS AND SYNTHESIS OF rRNA DURING DEVELOPMENT

In amphibian embryos the size of the nucleolus correlates with the rate of synthesis of rRNA. Nucleoli appear for the first time at gastrulation, the stage at which rRNA synthesis begins in the embryo (16). Nucleoli gradually increase in size up to tail-bud stage concomitant with an increased rate of rRNA synthesis. Barr and Esper (23) noted that the single nucleolus of heterozygote embryos is as large as the combined

volumes of the two nucleoli in wild-type cells. We showed (21) that the 1-*nu* and 2-*nu* embryos synthesize identical amounts of rRNA during early development.

The correlation of nucleolar appearance and size with the rate of rRNA synthesis is unique. In direct contrast, the synthesis of both dRNA and 4S RNA begins in embryos of *X. laevis* before nucleoli are visible (11). Furthermore, their rates of synthesis are exceedingly high during late cleavage just prior to gastrulation and gradually decrease with subsequent development as the nucleolus is enlarging in size and the synthesis of rRNA is increasing.

Any molecular or cytological event which can be shown to occur in a wide variety of developing embryos will have correspondingly greater interest in biology. An ubiquitous event is the delayed onset of both visible definitive nucleoli and rRNA synthesis in developing embryos. A wide variety of embryos does not contain detectable nucleoli during early development, and several have been shown not to synthesize rRNA during early embryogenesis (11). A list of animals is presented in table 3 along with the developmental stage of the embryo when nucleoli are first discernible.

TABLE 3.—Appearance of the nucleolus during development*

Category	Genus	Stage of appearance	Refer- ence
Mollusc.....	<i>Limnaea</i>	24-cell stage.....	(24)
	<i>Helix</i>	Blastula.....	(25)
	<i>Chiton</i>	Prototroch, Apical Tuft.....	(26)
	<i>Cyclops</i>	2-8 cell stage.....	(27)
Tunicate.....	<i>Ciona</i>	Neurula.....	(28)
	<i>Ascidia</i>	Metamorphosis.....	(29)
Echinoderm...	Sea urchin.....	Mesenchyme blastula.....	(30)
Insect.....	<i>Chironomus</i>	Blastoderm.....	(31)
	<i>Drosophila</i>	Blastoderm.....	(32)
Amphibian....	<i>Triturus</i>	Gastrula.....	(33)
	<i>Rana</i>	Gastrula.....	(14)
	<i>Xenopus</i>	Gastrula.....	(34)
Fish.....	<i>Fundulus</i>	Gastrula.....	(35)
	<i>Coregonus</i> (whitefish).....	Gastrula.....	(36)
	<i>Misgurnus</i> (loach).....	Mid-blastula.....	(37)
Bird.....	Chicken.....	Late blastula.....	(38)
Mammal.....	Mouse.....	2-cell stage.....	(39)
	Rat.....	8-cell stage.....	(40)

*Dr. A. A. Neyfakh provided the references to Russian articles as well as those related to fish and chicken embryos.

STAGE-DEPENDENT SYNTHESIS OF rRNA AND APPEARANCE OF NUCLEOLI

The pattern of rRNA synthesis during oogenesis and development is *stage dependent*. It is not dependent on the number of active chromosomes, since heterozygous (1-*nu*) *X. laevis* embryos synthesize rRNA at the same rate as wild-type embryos (21). Furthermore, the heterozygote has synthesized the same number of ribosomes during oogenesis as the wild type, since the unfertilized eggs of heterozygote females have the same quantity of rRNA as normal eggs. We do not know whether there is an alteration in nucleolar number in oocytes which are heterozygous for this mutation. The rate of synthesis of rRNA does not depend on the number of cells, since haploid embryos having twice as many cells as diploid embryos at each stage of development make ribosomes at the same rate as the diploid embryo (41).

The synthesis of rRNA is regulated in some way by the cytoplasm of the cell. This has been shown for embryos of *X. laevis* by nuclear transplantation (17).

Endoderm nuclei from embryos of different stages can be transplanted into enucleated unfertilized eggs of *X. laevis* and a certain proportion will support normal development (42). We have asked the following questions of this system. If nuclei from tissues synthesizing predominantly rRNA and having prominent nucleoli are transplanted into unfertilized eggs, what would be the effect on rRNA synthesis and on the nucleoli themselves? Would the pattern of RNA synthesis revert to that of the normal cleaving embryo (no rRNA synthesis) or would the transplanted nucleus, previously supporting largely rRNA synthesis during cleavage, give rise to daughter nuclei that now support the synthesis of rRNA? The results shows that embryos derived from transplanted nuclei have a pattern of RNA synthesis indistinguishable from normal embryos; that is, when the embryos progress to the late cleavage stage they synthesize dRNA, but not rRNA. At the onset of gastrulation, rRNA synthesis recommences as in normal embryos. Due to the relative insensitivity of the technique, it is impossible to measure how long rRNA synthesis can persist immediately after nuclear transplantation. However, within 40 minutes after transplantation, even before first cleavage, the nucleoli of the transplanted nucleus are no longer visible. From these experiments we conclude that the nucleolar structure is lost and synthesis of rRNA is inhibited by egg cytoplasm (17).

CONCLUSIONS

Our interest in the nucleolus lies in the fact that the presence, number, and even size of this organelle can be used to determine the presence, absence, and even to some extent rates of synthesis of a specific, and well-defined, set of gene products—ribosomal RNA. The many correlations

of nucleoli with rRNA give confidence to interpretation of purely cytological data on a molecular level. Thus embryos which for technical reasons are not suitable for direct chemical analysis of rRNA can be examined for number and size of their nucleoli. The cumulative information (table 3) shows that delayed ribosome synthesis (nucleolar appearance) is the rule of embryogenesis rather than the exception.

Our analysis of the unusual patterns of nucleoli and synthesis of rRNA during oogenesis and development has led to the following interpretation in terms of the functional role of ribosomes in protein synthesis (11). During oogenesis, intense synthesis and marked stability of ribosomes result in their accumulation and storage. When the egg is laid, it has a great excess of these particles relative to the other two RNA components of the protein-synthesizing machinery (transfer and messenger RNA). These ribosomes are conserved during development and are parceled out to daughter cells. The very low relative amount of transfer and messenger RNA per ribosome in the egg is corrected by intense synthesis of these two classes of RNA during embryogenesis when the synthesis of new ribosomes is minimal. By the time the embryo has reached the early swimming stage and differentiation has proceeded to the point that organ primordia have been formed, ribosome synthesis becomes more intense and the embryo begins to grow and rapidly uses its stored yolk for synthesis of new proteins. Therefore, ribosomes seem to be stored in oocytes for future use during development much like yolk, or glycogen. There is no doubt that the striking prominence of nucleoli in oocyte nuclei attests to their remarkable ability to synthesize ribosomes.

RESUMEN

Las siguientes observaciones de ovocitos y embriones en desarrollo confirman firmemente la relación existente entre la presencia y el tamaño de los nucleolos con la presencia y velocidad de síntesis del ARN ribosómico.

- 1) El número de nucleolos en los ovocitos no se corresponde con el de la ploidía esperada. El poco frecuente gran número o tamaño de los nucleolos en los ovocitos se correlaciona con la tasa extraordinariamente elevada de ARNr que es característica de los ovocitos en crecimiento.
- 2) Cuando el ovocito entra en meiosis, los nucleolos desaparecen y cesa la síntesis del ARNr.
- 3) El desarrollo precoz de muchos embriones de caracteriza por la ausencia tanto de nucleolos visibles como de síntesis de ARNr.
- 4) En los anfibios, el comienzo de la síntesis del ARNr se correlaciona exactamente con la primera aparición de nucleolos visibles en el estadio de gástrula. A medida que continúa el desarrollo, los nucleolos aumentan de tamaño y aumenta la tasa de síntesis de ARNr.
- 5) Un mutante anucleolado de *Xenopus laevis* no tiene nucleolos definitivos y no sintetiza ARNr.
- 6) Mediante trasplante nuclear se ha demostrado que el citoplasma de óvulos no fecundados y de embriones en segmentación puede inhibir tanto la síntesis del ARN como la aparición nucleolar.

- 7) Mientras que la síntesis del ARNr se correlaciona en forma precisa con la presencia y tamaño de los nucleolos, la síntesis del ARN semejante al ADN y el ARN 4S está completamente no relacionada con la presencia o tamaño nucleolar.

La elevada tasa de síntesis y la notable estabilidad de los ribosomas en los ovocitos da por resultado grandes números de ribosomas en el óvulo no fecundado en relación a las otras dos clases de ARN (ARN semejante al ADN y ARN 4S). Estos ribosomas mantienen la síntesis proteica a lo largo del desarrollo del anfibio hasta los primeros estadios de la natación. Durante el desarrollo precoz, la síntesis del ARN_d y el ARN 4S es tan intensa en relación con la síntesis ribosómica, que se corrige su deficiencia relativa en el óvulo. En esta forma, el embrión en desarrollo aumenta su capacidad de sintetizar proteínas.

REFERENCES

- (1) RAVEN, CHR. P.: *Oogenesis: The Storage of Developmental Information*. New York, Pergamon Press, 1962, pp 1-274.
- (2) EDSTRÖM, J.-E., GRAMPP, W., and SCHOR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. *J Biochem Biophys Cytol* 11: 549-557, 1961.
- (3) CALLAN, H. G., and LLOYD, L.: Lampbrush chromosomes of crested newts *Triturus cristatus* (Laurenti). *Phil Trans Roy Soc [Biol]* 243: 135-219, 1960-61.
- (4) BROWN, C. A., and RIS, H.: Amphibian oocyte nucleoli. *J. Morph* 104: 377-414, 1959.
- (5) GALL, J. G.: Chromosomes and cytodifferentiation. In *Cytodifferential and Macromolecular Synthesis* (Locke, M., ed.). New York, Academic Press Inc., 1963, pp 119-143.
- (6) EDSTRÖM, J.-E., and GALL, J. G.: The base composition of ribonucleic acid in lampbrush chromosomes, nucleoli, nuclear sap, and cytoplasm of *Triturus* oocytes. *J Cell Biol* 19: 279-284, 1963.
- (7) FICQ, A.: Metabolisme de l'oogenèse chez les amphibiens. In *Symposium on Germ Cells and Development*. Milano, Fondazione A. Baselli, 1960, pp 121-140.
- (8) IZAWA, M., ALLFREY, V. G., and MIRSKY, A. E.: The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc Nat Acad Sci USA* 49: 544-551, 1963.
- (9) BROWN, D. D., and LITINA, E.: Variations in the synthesis of stable RNA's during oogenesis and development of *Xenopus laevis*. *J Molec Biol* 8: 688-695, 1964.
- (10) DAVIDSON, E. H., ALLFREY, V. G., and MIRSKY, A. E.: On the RNA synthesized during the lampbrush phase of amphibian oogenesis. *Proc Nat Acad Sci USA* 52: 501-508, 1964.
- (11) BROWN, D. D.: RNA synthesis during early development. In *Developmental and Metabolic Control Mechanisms and Neoplasia* (Ward, D. N., ed.). In press.
- (12) DAWID, I. B.: Deoxyribonucleic acid in amphibian eggs. *J Molec Biol* 12: 581-599, 1965.
- (13) ROSSET, R., MONIER, R., and JULIEN, J.: Les ribosomes d'*Escherichia coli*. 1. Mise en évidence d'un RNA ribosomique de faible poids moléculaire. *Bull Soc Chim Biol (Paris)* 46: 87-109, 1964.
- (14) BROWN, D. D.: Unpublished data.

- (15) NIEUWKOOP, P. D., and FABER, J.: Normal Table of *Xenopus laevis* (Daudin). Amsterdam, North-Holland Publishing Co., 1956, pp 1-243.
- (16) BROWN, D. D.: RNA synthesis during amphibian development. *J Exp Zool* 157: 101-113, 1964.
- (17) GURDON, J. B., and BROWN, D. D.: Cytoplasmic regulation of RNA synthesis and nucleolus formation in developing embryos of *Xenopus laevis*. *J Molec Biol* 12: 27-35, 1965.
- (18) BROWN, D. D., and LITINA, E.: RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. *J Molec Biol* 8: 669-687, 1964.
- (19) ELSDALE, T. R., FISCHBERG, M., and SMITH, S.: A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp Cell Res* 14: 642-643, 1958.
- (20) FISCHBERG, M., and WALLACE, H.: A mutation which reduces nucleolar number in *Xenopus laevis*. In *The Cell Nucleus* (Mitchell, J. S., ed.). London, Butterworth, 1960, p 30.
- (21) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (22) WALLACE, H., and BIRNSTIEL, M.: Ribosomal cistrons and the nucleolus organizer. *Biochim Biophys Acta* 114: 296-310, 1966.
- (23) BARR, H. J., and ESPER, H.: Nucleolar size in cells of *Xenopus laevis* in relation to nucleolar competition. *Exp Cell Res* 31: 211-214, 1963.
- (24) RAVEN, CHR. P.: *Morphogenesis: The Analysis of Molluscan Development*. New York, Pergamon Press, 1958, p 76.
- (25) DAUWALDER, M.: Initiation of RNA synthesis and nucleolar modification during cleavage in *Helix aspersa*. *J Cell Biol* 19: 19A, 1963.
- (26) COWDEN, R. R.: A cytochemical investigation of oogenesis and development to the swimming larval stage in the Chiton, *Chiton tuberculatum* L. *Biol Bull* 120: 313-325, 1961.
- (27) KIKNADZE, I. I.: About the existence of nucleolus at early stages of cleavage. *Tsitologia* 5: 319-320, 1963.
- (28) MANCUSO, V.: Acidi nucleici dell' uovo in sviluppo di *Ciona intestinalis*. *Acta Embryol Morphol Exp (Palermo)* 2: 151-162, 1959.
- (29) COWDEN, R. R., and MARKERT, C. L.: A cytochemical study of the development of *Ascidia nigra*. *Acta Embryol Morphol Exp (Palermo)* 4: 142-160, 1961.
- (30) COWDEN, R. R., and LEHMAN, H. E.: A cytochemical study of differentiation in early echinoid development. *Growth* 27: 185-197, 1963.
- (31) BEERMANN, W.: Der Nukleolus als lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.
- (32) DAS, C. C., KAUFMANN, B. P., and GAY, H.: Histone protein transition in *Drosophila melanogaster*. II. Changes during early embryonic development. *J Cell Biol* 23: 423-430, 1964.
- (33) KARASAKI, S.: Electron microscopic studies on cytoplasmic structures of ectoderm cells of the *Triturus* embryo during the phase of differentiation. *Embryologia (Nagoya)* 4: 247-272, 1959.
- (34) WALLACE, H.: Cytological and biochemical studies of anucleolate *Xenopus* larvae. *Quart J Micr Sci* 103: 25-35, 1962.
- (35) RICHARDS, A., and PORTER, R. P.: Analysis of early development of fish embryos by means of the mitotic index. II. The mitotic index in pre-neural tube stages of *Fundulus heteroclitus*. *Amer J Anat* 56: 365-393, 1935.
- (36) RICHARDS, A., and SCHUMACHER, B. L.: Analysis of early development of fish embryos by means of the mitotic index. III. The mitotic index of young embryos of *Coregonus clupeiformis*. *Amer J Anat* 56: 395-408, 1935.
- (37) PANKOVA, N. B.: The changes in cell nucleus at early stages of development of the loach, *Misgurnus fossilis*. *Tsitologia* 5: 36-42, 1963.

- (38) BECHTINA, O. G.: Early stages of cleavage in chick embryo. *Archiv Anat (Strasb)* 38: 77-85, 1960.
- (39) MINTZ, B.: Synthetic processes and early development in the mammalian egg. *J Exp Zool* 157: 85-100, 1964.
- (40) AUSTIN, C. R.: Nucleic acids associated with nucleoli of living segmented rat eggs. *Exp Cell Res* 4: 249-251, 1953.
- (41) BROWN, D. D., and GURDON, J. B.: Unpublished data.
- (42) GURDON, J. B.: The transplantation of living cell nuclei. In *Advances in Morphogenesis* (Abercrombie, M., and Brachet, J., eds.). New York, Academic Press Inc., 1965, vol 4, pp 1-43.

DISCUSSION

Sirlin: I remember from reading Brachet years ago that the earliest recognizable nucleoli occur at late blastula in the amphibian, not in the early gastrula. True, they are minute things but they can still be recognized. The fashion now seems to be to see them only from gastrulation onward. I wonder if someone recalls Dr. Brachet's observation. Perhaps you, Dr. Ficq? How early do you see something that really looks like a nucleolus, however small?

Ficq: I think that really it is after the blastula stage, but this observation was made in another species, not *Xenopus*. However, nucleoli may appear at earlier stages when cleavage is blocked by antimitotic agents.

Brown: During late blastula there are bodies in the nucleus which have been called "blobs." They resemble the blobs of the anucleolate embryo. One of their characteristic features is that they are not present in the genetically determined number. Bodies of this kind have also been described in mammalian embryos during early stages. Perhaps these are what you mean.

Sirlin: Could these account for a first phase of synthesis in the nucleolus of transfer RNA (tRNA), succeeded later by a phase of rRNA synthesis?

Brown: Please don't misunderstand me. Perhaps tRNA is formed in nuclear particles, but I think what we have been calling a nucleolus simply doesn't correlate with tRNA synthesis. No one knows where tRNA is made. Maybe it is made in some visible nuclear structure, one of these little bodies. However, the synthesis of tRNA does not at all correlate with the presence of typical nucleoli.

Waddington: I was going to mention the same point, and I should like to say that after listening to this discussion, it seems to me there is nothing against the suggestion that some sort of nucleolus, or anlage of the nucleolus, first appears in the late blastula, and for a time does nothing but tRNA synthesis; later on it grows bigger, when it starts doing the ribosomal synthesis, and at that time you recognize it as a really typical nucleolus.

Brown: Do you wish to say that anucleolate *Xenopus* cells have an anlage of a typical definitive nucleolus?

Waddington: I don't think one has evidence to deny this possibility. There are a number of blobs, and whether one of these blobs is an anlage of a definitive nucleolus, I am not prepared to say. Can we leave this subject and go on to another? Isn't it significant that the only embryos we know in which visible nucleoli occur at an extremely early stage are those of mammals, which don't have yolk and almost certainly have to immediately begin protein synthesis, and therefore really need ribosomes from the start?

Brown: I agree completely. The large yolky embryos have ribosomes stored during oogenesis like they have stored yolk or glycogen. There is no reason to assume that all embryos will have stored ribosomes to this extent. The stored ribosomes in the amphibian egg support all the protein synthesis of the embryo up to the time

they begin to grow. It is exactly at the time when they begin to grow that they need new ribosomes.

Mandel: In relation to the problem of tRNA synthesis in nucleoli, which has been discussed, two questions arise. First, is there an increase of tRNA in the *Xenopus* mutant without nucleoli? And second, if this does not occur, how do you explain the synthesis of proteins during the first steps in the development of the embryo?

Brown: The mutant embryos synthesize tRNA at the same rapid rate as normal embryos.

Wallace: Using pyronine staining, I have seen RNA-containing structures in *Xenopus* as early as the late blastula stage, but this does not detract from Dr. Brown's argument at all. I don't think they have anything to do with nucleoli. They correspond, I believe, to what I call blobs in the anucleolate mutants, that is to say, they may not be concerned with either nucleoli or rRNA synthesis. So I even agree with this detail in Dr. Brown's very nice analysis of RNA synthesis. I would like to take issue with him on one minor point he didn't enlarge upon in his talk. He showed one slide depicting not only normal *Xenopus* tadpoles and the mutant anucleolate tadpoles but also what he has described before as phenocopies produced by culturing in a medium lacking magnesium. Now I'd like to state categorically that these are not phenocopies of the anucleolate mutant. I could pick them out every time. So could each of you with a little experience, and I believe Dr. Brown could. Can you?

Brown: These are morphological phenocopies to the extent that they arrest at the same stage as the mutant (Brown and Gurdon, Proc Nat Acad Sci USA 51: 139, 1964). At this stage the normal embryo begins to take up magnesium from the medium; a large percentage of this magnesium is associated with newly synthesized ribosomes.

Wallace: I talked to Dr. Gurdon about this, and I hope I quote him correctly. He told me that they have nucleoli.

Brown: That's right. They make rRNA also.

Wallace: So they are not phenocopies.

Brown: They are phenocopies because they are dying for related reasons and at the same stage. A magnesium-starved embryo might die because new ribosomes sequester magnesium which is needed for critical enzymatic reactions.

Perry: Two points. First, a comment in regard to the early appearance of the nucleolus in the mammals: On your table you showed a two-cell stage. I happen to be fairly familiar with the work of B. Mintz (J Exp Zool 157: 85-100, 1964) in which she has investigated RNA synthesis in mouse embryos during early developmental stages. Actually, at the two-cell stage I don't believe that there is really a nucleolus as such. There is a heterochromatic shell which becomes only very lightly labeled when one incubates the embryos with radioactive RNA precursors. And then at the four-cell stage one gets an appearance of real definitive-type nucleoli. However, the RNA labeling of the nucleolus is still predominantly peripheral at the eight-cell stage. Furthermore, an interesting correlation emerged when Mintz compared the sensitivity to actinomycin D at the early stage, when there is essentially no nucleolus, and at later stages. At the two-cell stage RNA synthesis is relatively insensitive to actinomycin, whereas in the later stages it is quite sensitive, and one observes a morphological regression of the nucleolus to an earlier state. The effect on the cytoplasmic labeling is also much greater in the later stages.

Brown: I want to emphasize that there are cases in which nucleoli are present but not synthesizing rRNA, such as the mature amphibian oocyte in which the nucleoli were synthetically active at one time but now are inactive. However, I know of no case of a cell without a nucleolus which is synthesizing rRNA.

Perry: And a question: Just what are your speculations about the ways the implantation experiments might be explained? That is, what are the mechanisms by which you can implant a nucleus that contains a nucleolus into an egg and have the

nucleolus disappear? What do you think might be the feedback mechanism involved? Have you any ideas on this?

Brown: In developing embryos, the rates of rRNA synthesis are stage dependent. These transplantation experiments are just another demonstration of this nuclear-cytoplasmic interaction, but on a molecular level. The cytoplasmic control has been shown in a more positive way by Graham and Gurdon (personal communication) who have transplanted nuclei with H^3 -thymidine into unfertilized *X. laevis* eggs. Even nuclei with very long division times begin synthesizing DNA shortly after transplantation. In other words, synthesis of RNA and DNA by these nuclei resembles that of the fertilized egg and cleaving embryos. To me this represents a potential assay system with which your question could be studied. However, we can't answer it yet.

Ritossa: In regard to your transplantation experiments, I would like to know whether the cytoplasm of unfertilized eggs has a preferential inhibition on the different classes of RNA. Namely, when you transplant a nucleus, is all the RNA synthesis blocked or are some classes of RNA inhibited and other classes still synthesized?

Brown: The early embryos derived from transplanted nuclei synthesize predominantly dRNA just like normal cleaving embryos. We have not compared this dRNA with that of normal embryos by hybridization, so we don't know if it represents transcription of the same genes. It is the same in sedimentation properties and base composition as dRNA from normal cleaving embryos and the embryo will differentiate in the same manner as the controls. These comments suggest that these nuclei must be transcribing the same genes as control nuclei and therefore the changes which occur must be very specific ones.

Ritossa: One more question. Have you shown that isolated nuclei can continue RNA synthesis *in vitro*?

Brown: We have not studied RNA synthesis in isolated nuclei.

Schreiber: A short remark about the first appearance of the nucleolus during segmentation. There is a great variety of segmentation in the mollusks, and I can assure you that in the early blastomeres, the first quartets of the spiral segmentation of the snail, there is a very nice, visible nucleolus, at least in the macromeres. So I think that this very interesting study should also be extended to other categories of invertebrates to determine if the same thing occurs in these different species.

Brown: There are other mollusks which don't have nucleoli visible right away. There is such a wide variety of embryos without visible nucleoli during early stages that yours may be the exception rather than the rule.

Waddington: May I ask Dr. Schreiber whether his mollusk is one of the kind in which the egg is enclosed in a capsule containing a lot of proteinaceous material which is absorbed, so that the egg starts growing immediately. Some molluscan eggs begin to increase in mass during the cleavage period and are therefore more comparable to the mammalian egg than to the amphibian egg; they would therefore be expected to have nucleoli at an early stage.

Schreiber: I am working with *Australorbis glabratus* Say, a Planorbis mollusk that has a large reserve of materials in the egg capsule. This material is metabolized by the large, polyploid "endormeres" which form the so-called "larval liver" as soon as they invaginate at the blastopore.

Metabolism of the Nucleolus During Oogenesis, Maturation, and Early Steps of Embryonic Development in Echinoderms^{1, 2}

ADRIENNE FICQ,³ *Laboratoire de Morphologie Animale, Université Libre de Bruxelles, Brussels, Belgium*

SUMMARY

Experiments were designed to follow the fate of the heavily labeled RNAs of the nuclear sap and the nucleoli of the oocyte at maturation time, when the germinal vesicle breaks down. In these experiments, fragments of Echinoderm ovaries (sea urchin and starfish) were treated with a radioactive RNA precursor (uridine and cytidine). The maturation of the oocytes was then artificially induced, and the localization of the

RNAs, synthesized in the nuclei and released into the cytoplasm by the rupture of the nuclear membrane, could then be followed by autoradiography. The results showed that, at least as far as can be judged by optical microscopy, the radioactivity was evenly distributed throughout the cytoplasm of the unfertilized egg.—*Nat Cancer Inst Monogr* 23: 311–323, 1966.

THE GROWING OOCYTE is one of the favorite materials for the study of nucleolar metabolism. More than 20 years ago, it was shown that the large number of nucleoli or the size of a single nucleolus is directly linked to the high synthetic activity of this cell (1). The relationship between the nucleolus and the synthesis of ribosomal ribonucleic acid (rRNA) is now a well-established fact (2-5). In the growing oocyte, the development of nucleoli is linked to the elaboration of the ribosomes and their storage. On the other hand, it is well known that, at the time of maturation, when nucleoli disappear as a result of the breakdown of the germinal vesicle, the ribosomes of the sea urchin oocyte become metabolically inert (6, 7). Ribosomal activity for protein synthesis is resumed shortly after fertilization (8) or parthenogenetic activation (9, 10). Such a protein synthesis is a prerequisite for normal mitotic activity during cleavage. In the presence of puromycin, segmentation of the egg stops (11).

¹Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

²A part of this work was done at the Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy.

³We wish to thank Euratom (contract 016-61-10 ABIE) for financial support and Merck Sharp & Dohme, Rahway, N.J., for the generous gift of actinomycin D.

Several experimental results (9, 10, 12) have led to the hypothesis that preformed ribosomes or pre-existing messenger RNAs (mRNAs) are implicated in protein synthesis concomitant to cleavage. It seems, thus, that during segmentation, no new ribosomes are synthesized (13, 14) but rather that ribosomes, momentarily inhibited at the time of maturation, are reactivated by a mechanism which might be called derepression.

The work of Maggio *et al.* (12) has shown that protein synthesis is stimulated in the ribosomes of unfertilized sea urchin eggs after treatment with trypsin. Such a phenomenon might represent a model for ribosome activation.

Actual rRNA synthesis does not take place in the sea urchin embryo before more advanced stages (blastula or gastrula) (14, 15), when nucleoli reappear in the nuclei.

It has been shown that, in animal cells, treatment with fluorodeoxyuridine induces the persistence of the nucleoli (16). Nucleoli, therefore, seem to appear and to persist when there is no DNA synthesis; this is also true for the oocyte. But in the cleaving egg, in which mitoses occur at fast rhythm, nucleoli are absent as long as DNA synthesis is active. At that early stage, the embryo utilizes cytidine and uridine, besides thymidine, for such a synthesis (17, 18). The synthetic activity of nucleolar RNAs during oocyte growth and of nuclear sap RNAs in young oocytes has been studied in many species (19-24). Let us mention, in particular, a very recent paper of Gross *et al.* (25) about RNA synthesis during oogenesis in *Arbacia punctulata*. After several days of labeling, the gonads were collected and analyzed by autoradiography and sedimentation in sucrose gradient. Most of the radioactivity in RNA from mature eggs was found in the ribosomal RNA. Radioactive heterogeneous RNA could also have been present.

It seemed worthwhile to study the fate of the strongly labeled RNAs at the time when the germinal vesicle breaks down. Such observations could perhaps give information on the localization of the stable mRNA which is probably present in unfertilized and mature eggs (26, 27). The experiments to be described in this paper were designed to see whether these RNAs have a particular localization or whether they are evenly distributed throughout the cytoplasm of mature and unfertilized eggs. After treatment of fragments of ovaries with a radioactive RNA precursor, the maturation of the oocytes was artificially induced (figs. 1, 2, and 3). The localization of the RNAs synthesized in the nuclei and released into the cytoplasm by the rupture of the nuclear membrane was then followed by autoradiography.

MATERIALS AND METHODS

All experiments were similarly performed at the same time *in vivo* and *in vitro* on *Arbacia lixula* and *Paracentrotus lividus* (January and Feb-

ruary) and *Asterias rubens* (October, December, and March).⁴ The eggs spawned at the beginning of the experiment were discarded. The others were treated in the following manner:

A. *Labeled for 1 hour*.—The eggs were then divided into 2 fractions: 1) unfertilized eggs (half of the sample centrifuged at 15,000 rpm for 5 minutes in 0.75 M sucrose); 2) unfertilized eggs which were then fertilized and fixed after 1 hour (acetic acid, alcohol, 3:1). Fragments of ovaries were fixed: (a) as such; (b) after 2 hours in hypotonic sea water (60% sea water, 40% distilled water) (28); (c) after 2 hours in sea water added with CaCl₂ (60% sea water, 40% CaCl₂ isotonic for sea water) (29).

B. *Labeled for 14 hours*.—Six series of experiments, with the same types of samples as in A, were performed:

- (1) 2 treatments with uridine-³H
specific radioactivity: 2.44 c/mm, 50 μ c / ♀ *in vivo*, 5 μ c/ml *in vitro*
- (2) 2 treatments with cytidine-³H
specific radioactivity: 2.02c/mm, 50 μ c / ♀ *in vivo*, 5 μ c/ml *in vitro*
- (3) L-methionine-¹⁴CH₃
specific radioactivity: 6.28 mc/mm, 50 μ c / ♀ *in vivo*, 5 μ c/ml *in vitro*
- (4) L-leucine-¹⁴C
specific radioactivity: 10.7 mc/mm, 25 μ c / ♀ *in vivo*, 3 μ c/ml *in vitro*

The above chemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England. Moreover, in the 4 experiments with uridine-³H and cytidine-³H the same runs were made in the presence of 20 μ g/ml actinomycin (Merck, Sharp & Dohme).

In the methionine experiments, enzymatic digestions of the sections by 0.1 mg/ml ribonuclease (Sigma Chemical Co., St. Louis, Mo.), were done at 37°C for 1 hour in distilled water.

The autoradiographic procedure used has been described elsewhere (30).

RESULTS

Incorporation of Uridine-³H

After a 1-hour pulse and a 2-hour chase, the nucleus and the nucleoli of the little oocytes at the periphery of the ovarian tubules remained labeled. Centrifugation did not displace this radioactivity. Several labeled maturation spots could be observed in the large oocytes (figs. 4 and 5).

When maturation was induced artificially, some labeled mature eggs appeared in the center of the acini, but in general, mature eggs and free

⁴ The best way to induce maturation in oocytes of *Asterias rubens*, consists in liberating them from the ovaries, pressing them gently with glass beads, and filtering them through nylon gauze. They are then released into normal sea water. Fertilization in this species is impossible in the laboratory.

unfertilized eggs scarcely incorporated uridine. Similar samples, in the presence of actinomycin, contained small oocytes in which incorporation was inhibited, chiefly in the nucleolus and to a lesser extent in the cytoplasm. Nuclear sap remained partially labeled. As in the controls, mature oocytes and unfertilized eggs were unlabeled.

After 14 hours of labeling and 1 hour of chase, both small and large oocytes were highly radioactive. Centrifugation displaced the labeling of the nuclear sap to the bottom of the germinal vesicle. Mature eggs were very weakly labeled or not at all. In the presence of actinomycin, the uptake of uridine was largely inhibited. In particular, in the small oocytes, the nucleoli were unlabeled. The residual activity of the nuclear sap surrounded the nucleolus.

In numerous cases, in the presence of actinomycin, maturation spots remained labeled, suggesting that the migration of the labeled material had been prevented. Unfertilized eggs, labeled for 1 or 14 hours, had very little activity, of the order of magnitude of the background which was rather high. This activity was not sedimented by centrifugation.

Incorporation of Cytidine-³H

After 1 hour of labeling, the nuclear sap and the nucleoli of the young and the large oocytes were highly radioactive (fig. 6). Some radioactivity was observed around the sections, probably due to the migration of a low molecular weight RNA. Mature eggs were weakly labeled but more than with uridine-³H. When maturation was induced, it seemed that the small oocytes lost their radioactivity in favor of the large ones during the stay in the cold medium. In mature eggs, several maturation spots were labeled.

After 14 hours of labeling, the small oocytes had lost their radioactivity. High labeling was observed in the nucleoli, the nuclear sap, and the cytoplasm of the large ones (fig. 7). When maturation was induced, some mature eggs were highly labeled (fig. 8). Unfertilized eggs, centrifuged or not, did not incorporate cytidine.

Protein Synthesis and RNA Methylation Studied by ¹⁴CH₃-Methionine

After 1 hour of labeling, uniform radioactivity was observed in the nuclei and the cytoplasm of all oocytes. Treating the sections with ribonuclease decreased this activity both in the nucleus and cytoplasm. Certain large oocytes were highly labeled in the center of the ovarian tubules; in particular, ribonuclease seemed to decrease that activity as if a significant degree of RNA methylation was taking place in these mature oocytes.

When maturation was induced in fragments of ovaries labeled for 1 hour, some labeling appeared in mature oocytes and in unfertilized eggs. After ribonuclease, this activity decreased strongly or disappeared, as did the background. Unfertilized eggs, if labeled for 1 hour and then fertilized and fixed 1 hour later, were exceedingly radioactive. Ribonuclease

labeled only a small part of this activity, and one may therefore conclude that it corresponds, essentially, to incorporation into proteins.

The picture obtained after 14 hours did not differ greatly from that obtained after 1 hour. Treating the sections with ribonuclease eliminated the greater part of the radioactivity in the large oocytes and in the mature eggs. As with short pulse labeling, unfertilized eggs which had been labeled for 14 hours were only weakly active. The same eggs, fertilized and fixed 1 hour later, were much more radioactive, and the major part of this activity remained present after treatment of the sections with ribonuclease.

Proteins Labeled by L-Leucine- ^{14}C

After 1 or 14 hours, the small oocytes were moderately labeled; maximal activity was found in the large oocytes whose nucleoli and nuclear sap were more strongly labeled than cytoplasm. Mature and unfertilized eggs were practically unlabeled.

After the induction of maturation, some mature eggs in the middle of the acini became relatively active. Unfertilized eggs, in contact with leucine- ^{14}C for 14 hours, were only slightly labeled. If fertilized and fixed 1 hour later, they became highly active. Centrifugation had no effect on the distribution of this activity.

CONCLUSIONS

With both RNA precursors (uridine and cytidine), the classical kinetics of incorporation are observed: first, labeling of the nucleolus, then of the nuclear sap after short pulses (1 hour), and labeling of the cytoplasm after longer periods of incubation (14 hours). A difference between the two precursors has, however, been noticed: Mature eggs are weakly labeled by cytidine under conditions in which they do not incorporate any uridine. This difference in behavior could, perhaps, reflect the fixation of terminal-CCA sequences on transfer RNA (tRNA).

In oocytes labeled just before maturation, it is the latter that induces the release of RNAs of nuclear origin throughout the cytoplasm. Even when the oocytes are centrifuged, the distribution of this RNA remains homogeneous. It seems, therefore, that the RNAs, synthesized in the oocyte nuclei, are not bound in the cytoplasm to specific particles, as far as one can judge by optical microscopy.

Our autoradiographic data are in good agreement with the results of Gross *et al.* (25) and with the biochemical observations (12) suggesting that mRNAs could be linked to ribosomes in sea urchin eggs.

In the series of experiments designed to study the methylation of RNAs by methionine- $^{14}\text{CH}_3$ and the incorporation of this amino acid into proteins, the following remarks are relevant: Protein synthesis is moderate in young oocytes, maximal in large oocytes, minimal in unfertilized eggs, and very high 1 hour after fertilization. Methylation occurs at a moderate rate in the young oocytes and becomes rather fast in the large ones. It

continues at a significant rate in mature eggs whose maturation is induced after labeling. But there is a striking difference between methylation of RNAs and incorporation of methionine into the proteins of eggs fertilized for 1 hour: While fertilization strongly stimulates protein synthesis, methylation remains a slow process in recently fertilized eggs. Control experiments, in which leucine- ^{14}C was used as a protein precursor, have shown that this amino acid behaves like methionine during oogenesis, maturation, and fertilization.

A more complete answer to these problems will probably emerge from the results of the electron microscope analysis at present being carried out.

RESUMEN

Se describen experimentos que fueron diseñados para seguir el destino de los ARNs del jugo nuclear marcados intensamente y los nucleolos de los ovocitos en el momento de la maduración, cuando se rompe la vesícula germinal.

Estos experimentos consistieron en tratar fragmentos de ovarios de Equinodermos (erizo de mar, estrella de mar) con un precursor radiactivo del ARN (uridina, citidina).

Se indujo entonces la maduración de los ovocitos en forma artificial pudiendo seguirse después, mediante autoradiografía, la localización de los ARNs sintetizados en los núcleos y liberados en el citoplasma por ruptura de la membrana nuclear.

Los resultados obtenidos muestran que, al menos por lo que puede juzgarse mediante microscopía óptica, la radiactividad se distribuye uniformemente a través del citoplasma del óvulo no fecundado.

REFERENCES

- (1) BRACHET, J.: La localisation des acides pentose nucléiques dans les tissus animaux et les oeufs d'amphibiens en voie de développement. *Arch Biol (Liege)* 53: 207-257, 1942.
- (2) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (3) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (4) EDSTRÖM, J.-E., GRAMPP, W., and SCHOR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. *J Biophys Biochem Cytol* 11: 549-557, 1961.
- (5) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (6) BRACHET, J.: Nucleic acids in development. *J Cell Comp Physiol* 60 (suppl 1): 1-18, 1962.
- (7) FICQ, A.: Metabolisme de l'oogenèse chez les amphibiens. *Sympos on Germ Cells and Development*. Fond A Baselli 121-140, 1960.
- (8) MONROY, A.: Incorporation of S35-methionine in the microsomes and soluble proteins during the early development of the sea urchin egg. *Experientia* 16: 114-115, 1960.
- (9) BRACHET, J., FICQ, A., and TENCER, R.: Amino acid incorporation into proteins of nucleate and anucleate fragments of sea urchin eggs: effects of parthenogenetic activation. *Exp Cell Res* 32: 168-170, 1963.

- (10) DENNY, P., and TYLER, A.: Activation of protein biosynthesis in non-nucleate fragments of sea urchin eggs. *Biophys Biochem Res Commun* 14: 245-249, 1964.
- (11) HULTIN, T.: The effect of puromycin on protein metabolism and cell division in fertilized sea urchin eggs. *Experientia* 17: 410-411, 1961.
- (12) MONROY, A., MAGGIO, R., and RINALDI, A. M.: Experimentally induced activation of the ribosomes of the unfertilized sea urchin egg. *Proc Nat Acad Sci USA* 54: 107-111, 1965.
- (13) BROWN, D. D., and LITINA, E.: RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. *J Molec Biol* 8: 669-687, 1964.
- (14) DECROLY, M., CAPE, M., and BRACHET, J.: Contribution à l'étude du métabolisme de l'acide ribonucléique au cours du développement embryonnaire. *Arch Int Physiol* 71: 129, 1963.
- (15) BROWN, D. D., and CASTON, J. D.: Biochemistry of amphibian development. I. Ribosome and protein synthesis in early development of *Rana pipiens*. II. High molecular weight RNA. *Develop Biol* 5: 412-434, 435-444, 1962.
- (16) HSU, T. C., HUMPHREY, R. M., and SOMERS, C. E.: Persistent nucleoli in animal cells following treatments with fluorodeoxyuridine and thymidine. *Exp Cell Res* 33: 74-77, 1964.
- (17) FICQ, A., and BRACHET, J.: Métabolisme des acides nucléiques et des protéines chez les embryons normaux et les hybrides létaux entre Echinodermes. *Exp Cell Res* 32: 90-108, 1963.
- (18) BIELAVSKY, N., and TENCER, R.: Incorporation of tritiated uridine into amphibian eggs. *Nature (London)* 185: 401, 1960.
- (19) FICQ, A.: Incorporation, *in vitro*, de glycocolle-1-C¹⁴ dans les oocytes d'Astéries. *Experientia* 9: 377-379, 1953.
- (20) ———: Etude autoradiographique du métabolisme des protéines et des acides nucléiques au cours de l'oogénèse chez les Batraciens. *Exp Cell Res* 9: 286-293, 1955.
- (21) ———: Effets de l'actinomycine D et de la puromycine sur le métabolisme de l'oocyte en croissance. Etude autoradiographique. *Exp Cell Res* 34: 581-594, 1964.
- (22) OZBAN, N., TANDLER, C., and SIRLIN, J. L.: Methylation of nucleolar RNA during development of the amphibian oocyte. *J Embryol Exp Morph* 12: 374-380, 1964.
- (23) DAVIDSON, E. H., ALLFREY, V. G., and MIRSKY, A. E.: On the RNA synthesized during the lampbrush phase of amphibian oogenesis. *Proc Nat Acad Sci USA* 52: 501-508, 1964.
- (24) ZALOKAR, M.: The role of nucleolus in the production of ribonucleic acid. *Genetics* 47: 996, 1962.
- (25) GROSS, P. R., MALKIN, L. I., and HUBBARD, M.: Synthesis of RNA during oogenesis in the sea urchin. *J Molec Biol* 13: 463-481, 1965.
- (26) BRACHET, J., DECROLY, M., FICQ, A., and QUERTIER, J.: Ribonucleic acid metabolism in unfertilized and fertilized sea-urchin eggs. *Biochim Biophys Acta* 72: 660-662, 1963.
- (27) BALTUS, E., QUERTIER, J., FICQ, A., and BRACHET, J.: Biochemical studies of nucleate and anucleate fragments isolated from sea-urchins eggs. A comparison between fertilization and parthenogenetic activation. *Biochim Biophys Acta* 95: 408-417, 1965.
- (28) DALCQ, A.: Recherches sur la physiologie de l'oeuf en maturation. *Arch Biol (Liege)* 33: 79-196, 1923.
- (29) ———: Recherches expérimentales et cytologiques sur la maturation et l'activation de l'oeuf d'*Asterias glacialis*. *Arch Biol (Liege)* 34: 507-674, 1924.
- (30) FICQ, A.: Autoradiography. *In* *The Cell* (Brachet, J., and Mirsky, A., eds.). New York, Academic Press Inc., 1959, vol I, chap 3, pp 67-91.

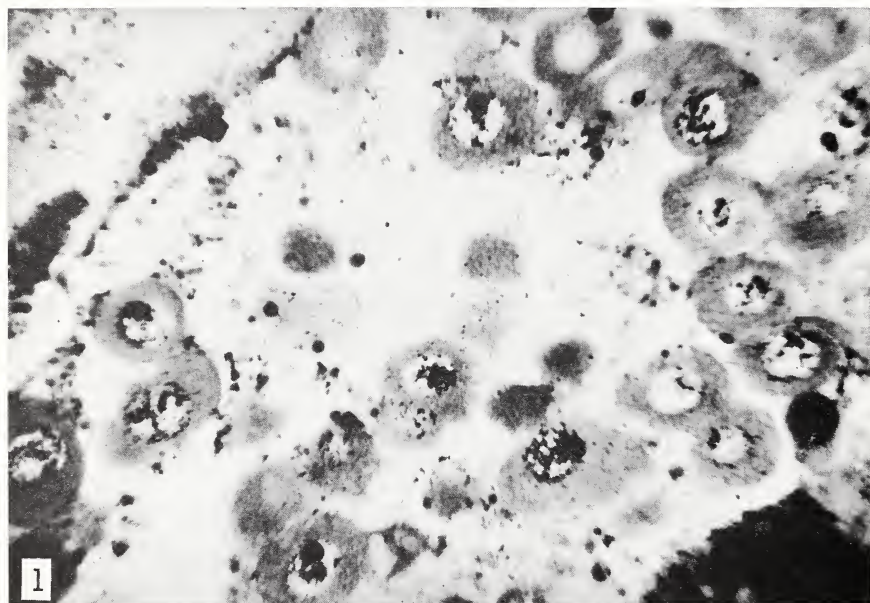
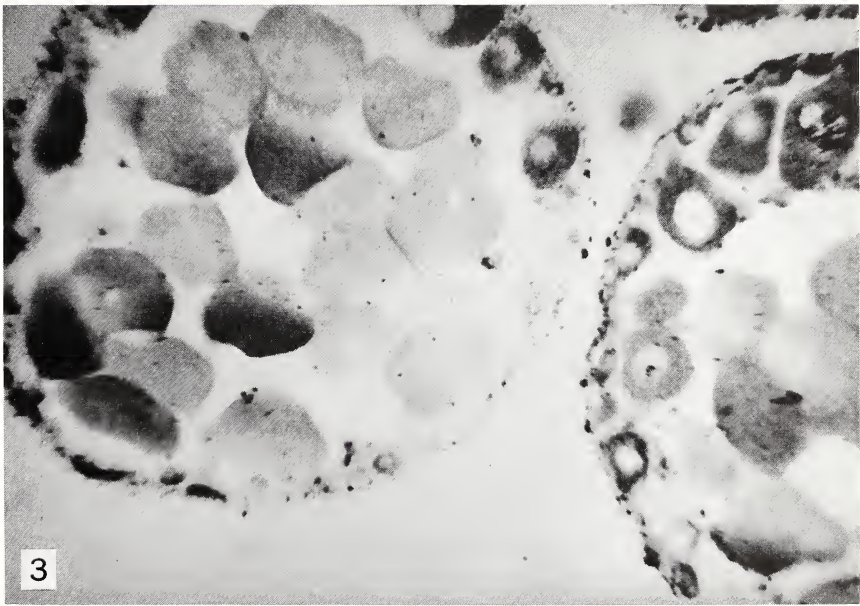
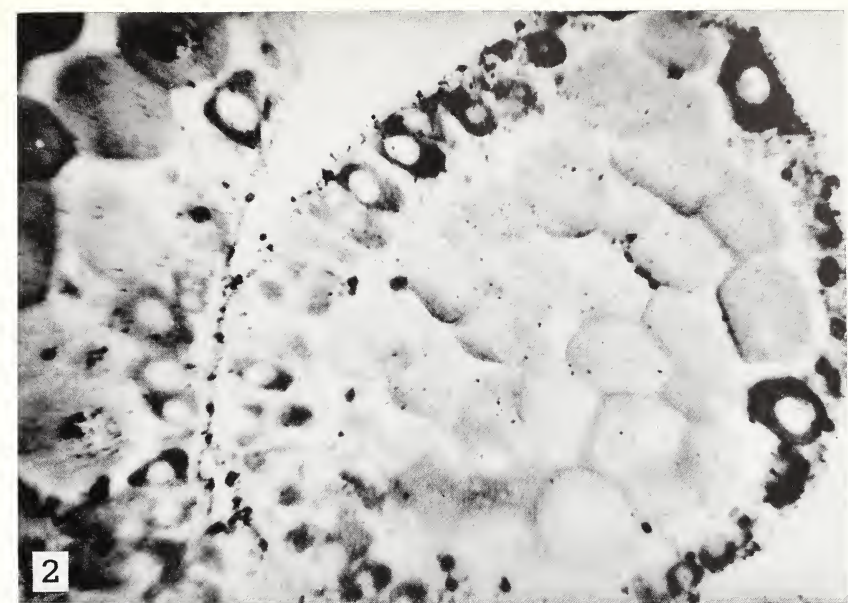
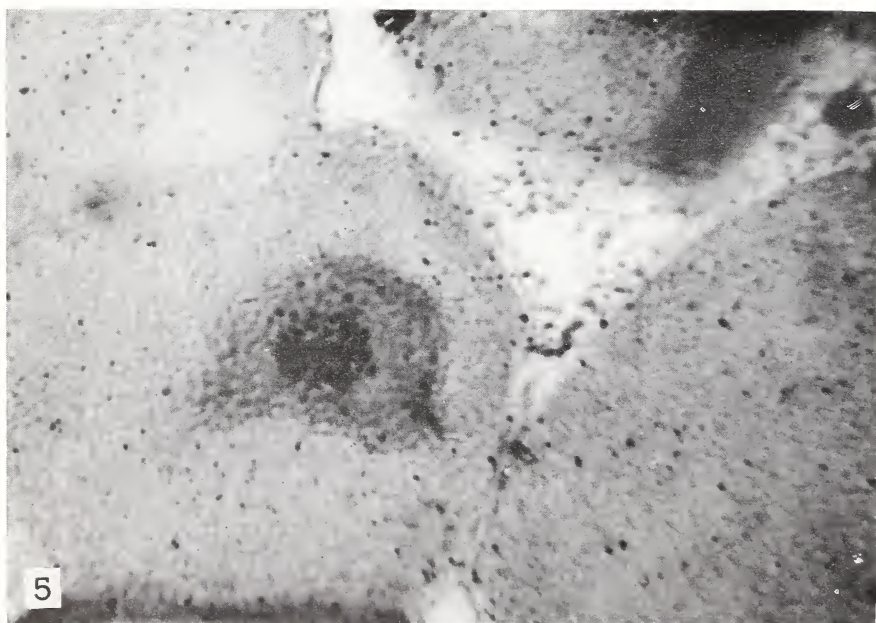
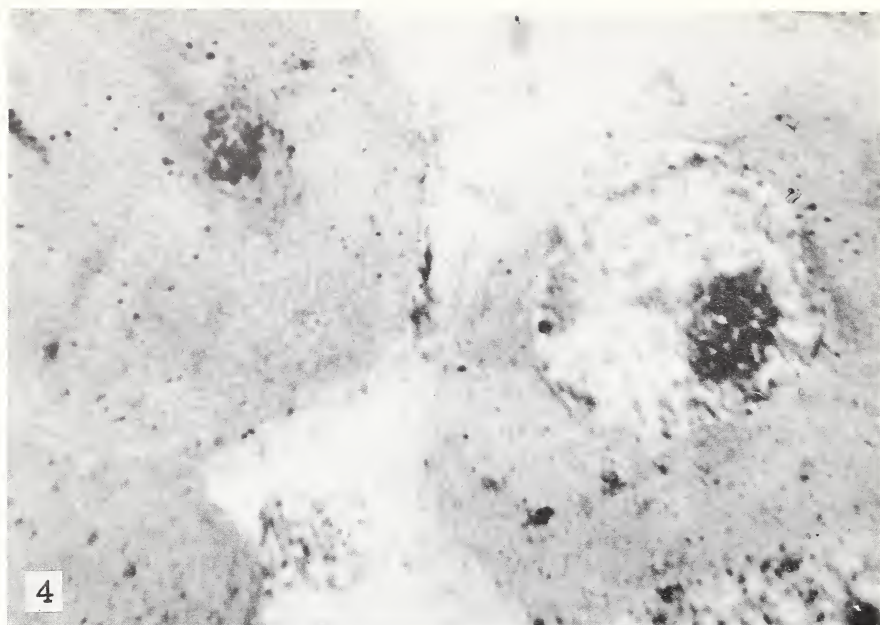


FIGURE 1.—Ovary of *Arbacia lixula*. Section through a normal acinus showing principally small and large oocytes.

FIGURE 2.—Ovary of *Arbacia lixula*, after induction of maturation. Section through an acinus showing principally mature eggs.

FIGURE 3.—Ovary of *Arbacia lixula* after centrifugation.





FIGURES 4 AND 5.—Sections of *Asterias rubens* oocytes. Maturation spots labeled with uridine- ^3H .

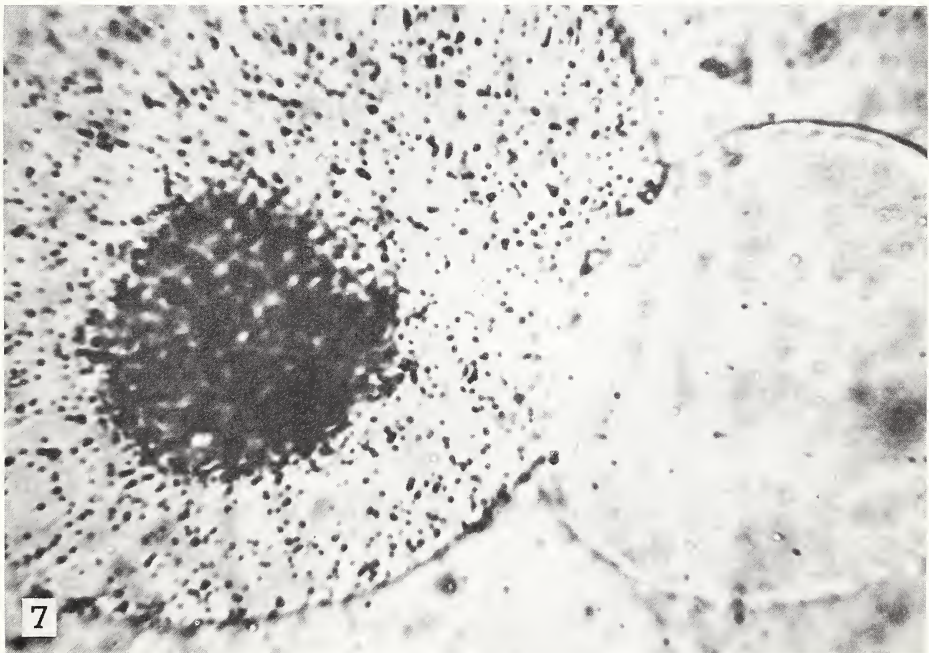
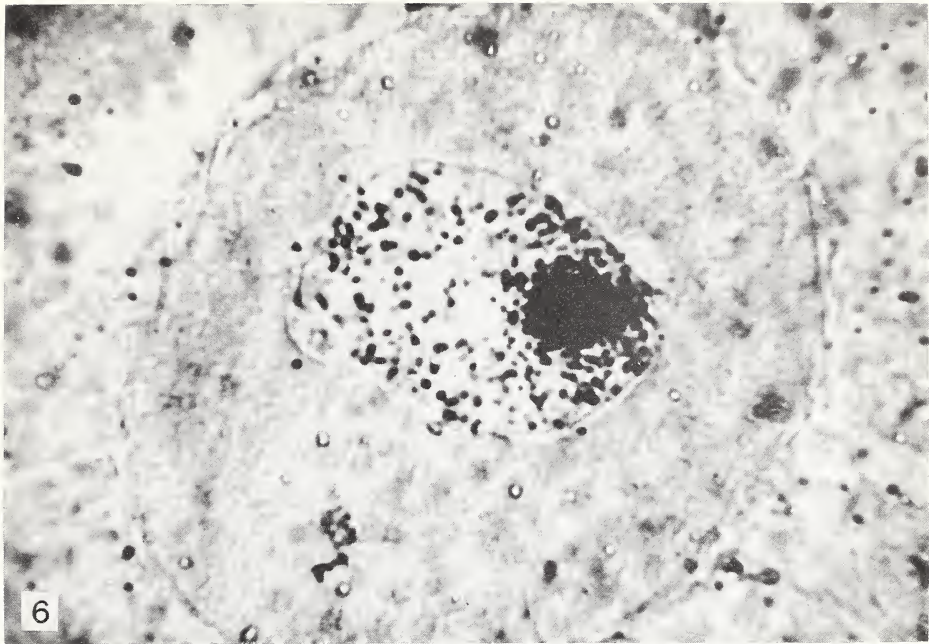


FIGURE 6.—Section through a large oocyte (*Asterias rubens*) after 1-hour incubation with cytidine-³H.

FIGURE 7.—Section through a large oocyte (*Asterias rubens*) after 14 hours' incubation; at right an inactive mature egg.

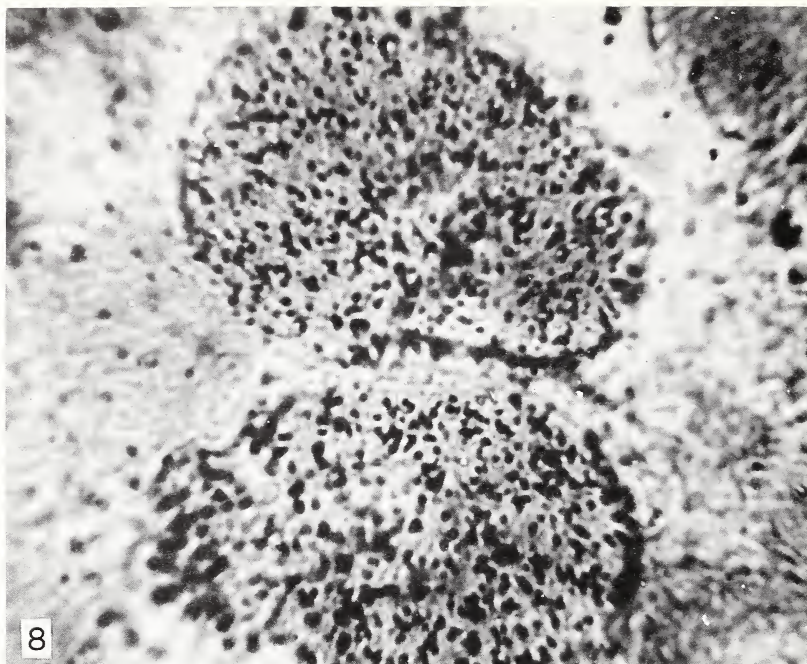


FIGURE 8.—Section of an ovary where maturation was induced. Some mature eggs are labeled in the center of the acinus.

DISCUSSION

Sirlin: I see that you have confirmed what we (Ozban, Tandler, and Sirlin, *J Embryol Exp Morph* 12: 373-380, 1964) found for the pattern of methylation during oogenesis in *Bufo*.

Ficq: I cannot quite agree with your results that the synthesis of tRNA is localized in the nucleolus, at least in urodeles. In the case of sea urchins, I can only say that RNA-methylating activity seems higher in mature oocytes than in eggs which have just been fertilized.

Feinendegen: Dr. Ficq, in what positions were the uridine and cytidine labeled?

Ficq: They were randomly labeled.

Synthesis of Basic Proteins and Cellular RNA Species During Sea Urchin Development^{1,2}

DONALD G. COMB and DAVID J. SILVER,³ *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*

SUMMARY

Some of the basic proteins synthesized from fertilization to the gastrula stage of sea urchin embryos appear to be histones with chromatographic properties similar to calf thymus histones. The first detectable synthesis of basic proteins associated with ribosomes occurred at the early pluteus stage. At specified levels of isotope and exposure time, RNA synthesis was first detected at the early blastula stage and only messenger RNA synthesis could be demonstrated at this time.

Transfer RNA synthesis was initiated at the time of gastrulation and newly synthesized transfer RNA was primarily associated with the ribosome fraction of cells. Ribosomal RNA synthesis could be first demonstrated at the early pluteus stage. The incorporation of methyl groups from ¹⁴C-methylmethionine into ribosomal RNA coincided with the first incorporation of ³²Pi.—*Nat Cancer Inst Monogr* 23: 325–336, 1966.

DURING DEVELOPMENT of the sea urchin embryo, protein synthesis is initiated at two distinct developmental stages (1,2). The first period of translation occurs immediately after fertilization and continues at an accelerated rate through cleavage. The proteins synthesized at this time are believed to be primarily proteins associated with the mitotic apparatus (3) and histones (4). Since little if any RNA synthesis can be detected during cleavage (5–7) and protein synthesis is insensitive to actinomycin, it has been proposed (8) that translation at this early period is in response to messenger RNA synthesized during oocyte maturation and stored in the ootid. The second period of translation occurs at the mesenchyme blastula stage. Many new enzyme activities appear at this time (9) as well as the

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This study was aided by Public Health Service grant GM-12632 from the National Institute of General Medical Sciences and grant GB-2349 from the National Science Foundation.

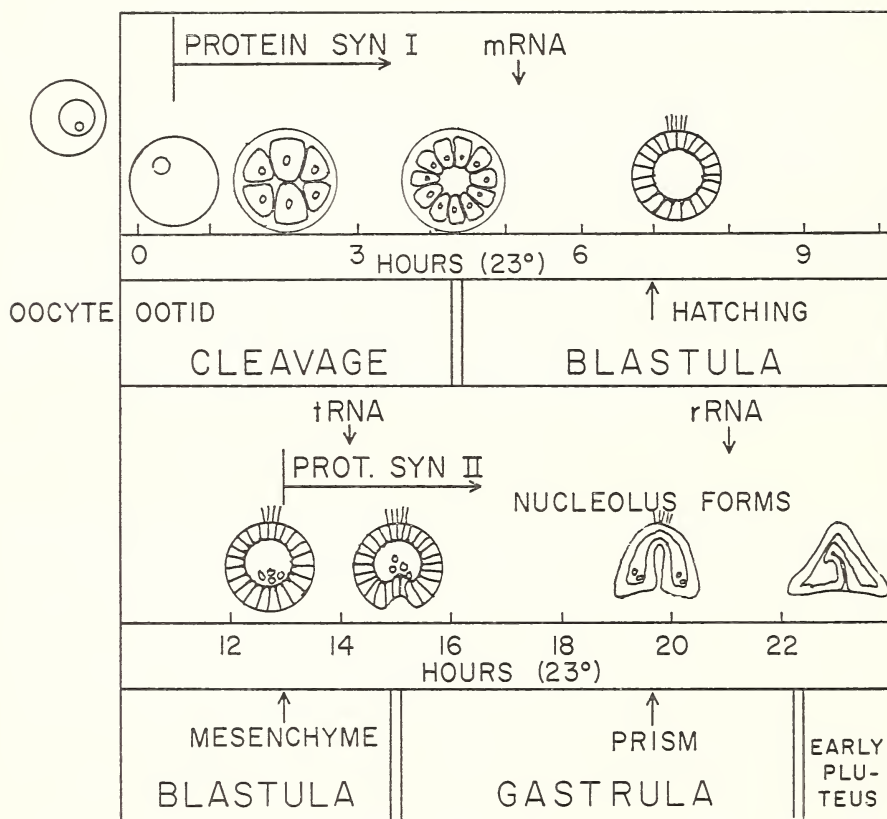
³ Much of this work was carried out at the Bermuda Biological Station and we would like to acknowledge the advice and assistance of Dr. W. H. Sutcliffe, Jr., Director of the Bermuda Biological Station.

first detectable adult protein (10). This second period of protein synthesis is undoubtedly in response to gene transcription at the blastula stage.

A sequential initiation of RNA synthesis can be demonstrated during development. The first detectable RNA synthesis occurs at the early blastula stage (5-7), although a low level of synthesis may possibly occur during cleavage (6). Only messenger RNA synthesis has been detected during the blastula stage of development (7, 11, 12).

Transfer RNA synthesis is initiated about the time of gastrulation (7, 13) and rRNA⁴ synthesis at the early pluteus stage (7, 12). This sequence of events is illustrated in text-figure 1.

Cytochemical studies indicate that the nucleolus first appears in the nucleus of the sea urchin embryo during the gastrula stage (14). Studies



TEXT-FIGURE 1.—Diagrammatic representation of sea urchin development with respect to time after fertilization at 23 C. Protein synthesis at the two stages indicated is discussed in the text. The arrows under the various types of RNA indicate the approximate time synthesis is first detected when embryos are exposed to specific levels of isotope for 3 minutes.

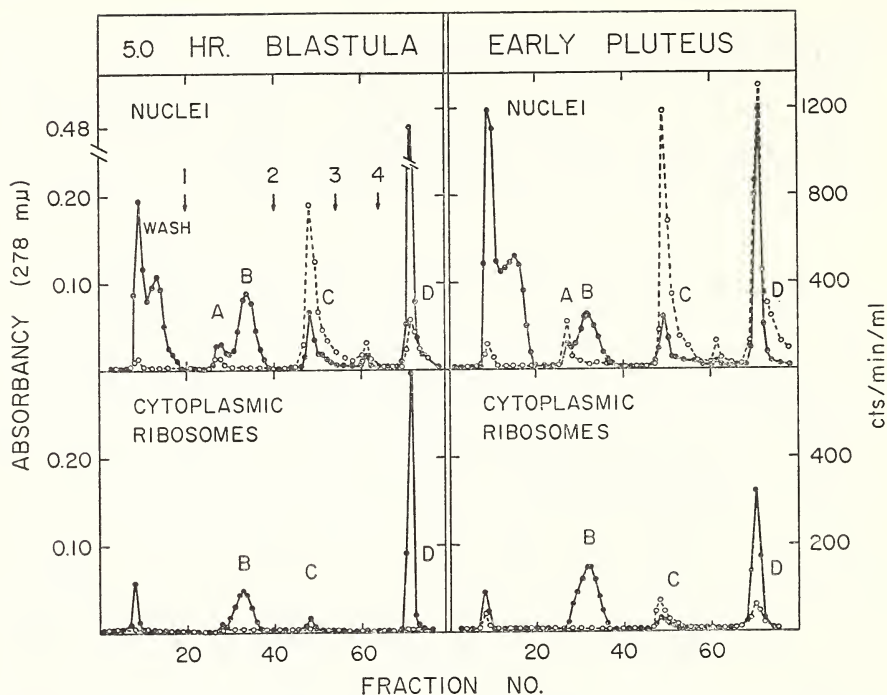
⁴ The following abbreviations are used: tRNA, transfer RNA; rRNA, ribosomal RNA; mRNA, messenger RNA; t-like RNA, transfer-like RNA.

from a number of laboratories (15-17) suggest the nucleolus to be the site of rRNA synthesis and possibly tRNA synthesis (18).

In the present studies we will (a) examine the types of basic proteins synthesized during early and late development of the sea urchin embryo, (b) demonstrate the sequential initiation of the various species of cellular RNA, and (c) attempt to evaluate the role of the nucleolus in these events.

BASIC PROTEIN SYNTHESIS DURING DEVELOPMENT

Sea urchin embryos (*Lytechinus variegatus*) at various stages of development were exposed to ^{14}C -alanine for 30 minutes, washed, and fractionated for nuclei and the microsome-ribosome fraction (7). The basic proteins were extracted from both preparations with 67% acetic acid (19), dialyzed, and chromatographed on carboxymethylcellulose in 6 M urea. Text-figure 2 compares the absorbancy and radioactivity profiles of proteins obtained from nuclei and ribosomes at an early and late stage of development. Ribosomes, believed to be of cytoplasmic origin, are present in the nucleus at all stages of development except the unfertilized egg (7). Four distinct protein peaks, labeled A through D, were present in the nuclear fraction. Comparison with the ribosomal basic proteins suggests that peaks B and D in the nuclear fraction are ribosomal proteins. Peaks A and C are believed to be histones, since most of the radioactivity was incorporated into these two peaks during early development and they co-chromatograph with calf thymus histone (text-fig. 3). Sea urchin peak C co-chromatographs with a calf thymus histone fraction which has a lysine-to-arginine ratio of 1.73 and probably corresponds to fraction IIb of Murray (20). Peak A of the sea urchin nucleus may represent lysine-rich histone but there is no direct proof of this other than its position of elution from the column. In addition, ^{14}C -labeled basic proteins from embryos at cleavage (3 hrs), blastula (7, 9, and 11 hrs), and gastrula (17 hrs) stages of development were chromatographed as described for 5-hour blastula and early plutei. The results indicated that nuclear peak C was highly labeled at all stages studied and nuclear peak A became progressively more labeled as development proceeded. At the pluteus stage a distinct switch in the labeling pattern is apparent (text-fig. 2). Approximately half of the ^{14}C -label entering the basic proteins is associated with peak D. The cytoplasmic ribosomal proteins are also labeled for the first time at this stage of development. However, the significance of this is questionable, since it represents only about 5% of that found in the nucleus, and may be due to nuclear contamination. A chase experiment at this stage would demonstrate whether newly synthesized ribosomal proteins are moving out of the nucleus, but this has not been done. The data demonstrate, however, that during the 30-minute exposure period, no detectable labeling of cytoplasmic ribosomal proteins occurred from early cleavage to the gastrula stage.

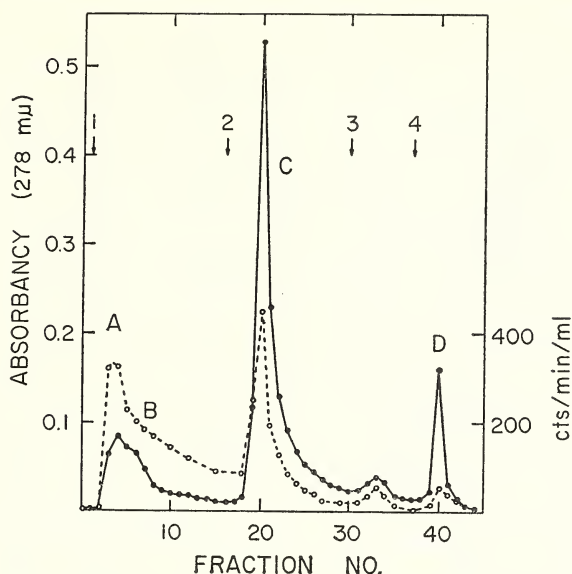


TEXT-FIGURE 2.—Carboxymethylcellulose chromatograms of basic proteins extracted from nucleic and from the microsome-ribosome pellet of the cytoplasm at the developmental stage indicated. The column size was 2×10 cm and all solvents contained 6 M urea. The sample was applied in 6 M urea and the column washed with 0.02 M sodium acetate, pH 4.2. The protein was eluted batchwise with the following solvents in the indicated order: 1) 0.1 M sodium acetate, pH 4.2; 2) 0.1 M sodium acetate, pH 4.2, 0.1 M sodium chloride; 3) 0.1 M sodium acetate, pH 4.2, 0.2 M sodium chloride; and 4) 6 M urea adjusted to pH 1.7 with HCl. Fractions of 7 ml were collected at room temperature. Embryos (5.2×10^6) were exposed to $10 \mu\text{C}$ of ^{14}C -L-alanine in 40 ml of sterile sea water for 30 minutes and the two fractions obtained as described in (7). —●—, absorbancy; ---○---, radioactivity.

RNA SYNTHESIS DURING DEVELOPMENT

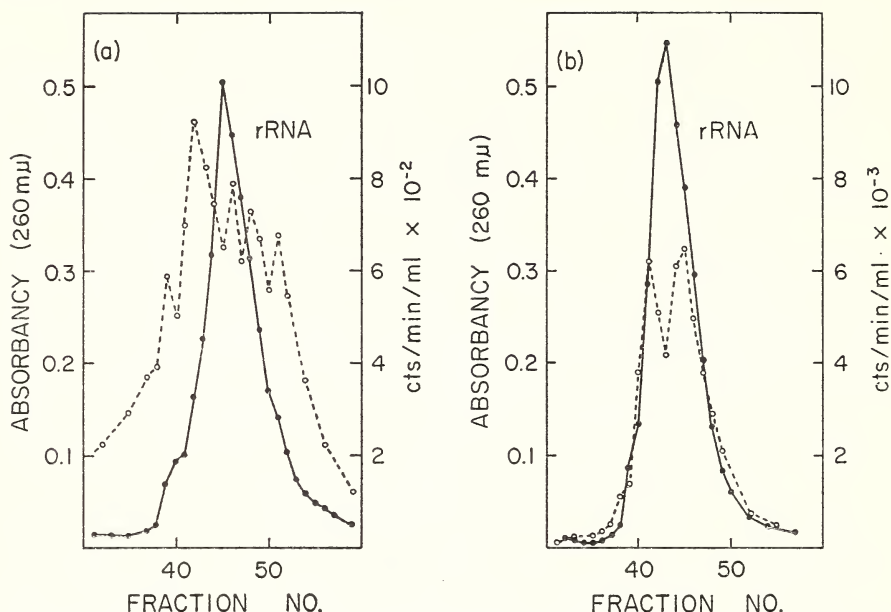
Messenger RNA

In the experiments reported we define mRNA as that fraction of rapidly labeled RNA 1) containing no methylated bases (no incorporation from ^{14}C -methylmethionine), 2) whose base composition resembles more closely that of DNA than that of rRNA or tRNA and, 3) that is heterogeneous and of high molecular weight by methylated albumin column chromatography. By short ^{32}P i-pulse experiments (3 min) we first detected the synthesis of mRNA at the early blastula stage. Radioactivity associated with mRNA was present in both the nuclear and microsomal fractions, but no radioactivity entered the free ribosome fraction (the



TEXT-FIGURE 3.—Co-chromatography of ^{14}C -labeled basic proteins extracted from 17-hour-gastrula nuclei with carrier calf thymus histone. The carboxymethyl-cellulose column size was 1×13 cm and elution of protein was carried out as described in text-figure 2. Fractions of 3 ml were collected. Carrier calf thymus histone (16 mg) and approximately 5 mg of sea urchin ^{14}C -basic protein were chromatographed. Peak D is due primarily to the sea urchin protein since only small amounts of a similar peak were detected when calf thymus histone was chromatographed alone. Tubes 19 to 25 were pooled for amino acid analysis; a lysine/arginine ratio of 1.73 was obtained. —●—, absorbancy; --○--, radioactivity.

pellet obtained between 22,000 and 105,000 $\times g$). After a longer pulse the microsome fraction became highly labeled (text-fig. 4). A similar pattern was obtained at either the blastula or gastrula stage. It is clear that the labeled RNA is very heterogeneous and resembles DNA in base composition (table 1). When embryos at the same two stages of development are exposed to ^{14}C -methylmethionine, there is no detectable incorporation of methyl groups into high molecular weight RNA—tRNA is methylated, however, at the gastrula stage (13). At the early pluteus stage the pulse-labeled RNA in the microsome fraction appears more homogeneous after chromatography and in contrast to earlier stages, label appears in the free ribosome fraction as well (7). The base composition of ^{32}P -RNA in the microsome fraction is shown in table 1. It appeared intermediate between that of mRNA and maternal rRNA. The reason for this shift to a more homogeneous population of RNA molecules is not clear. The two ^{32}P -labeled peaks may represent a mixture of mRNA and rRNA, as rRNA is first synthesized at this time.



TEXT-FIGURE 4.—Methylated albumin column chromatography of the microsome fraction of (a) gastrula embryos and (b) early plutei. Approximately 4×10^6 gastrulae were exposed to 0.5 mc of ^{32}P i in 40 ml of sea water for 5 minutes. The same number of plutei were exposed to 1.0 mc of ^{32}P i in 40 ml of sea water for 20 minutes. The microsome fraction is defined as that fraction of the cytoplasmic extract sedimenting between 750 and $22,000 \times g$ by centrifugation for 20 minutes. —●—, absorbancy; - - O - - -, radioactivity.

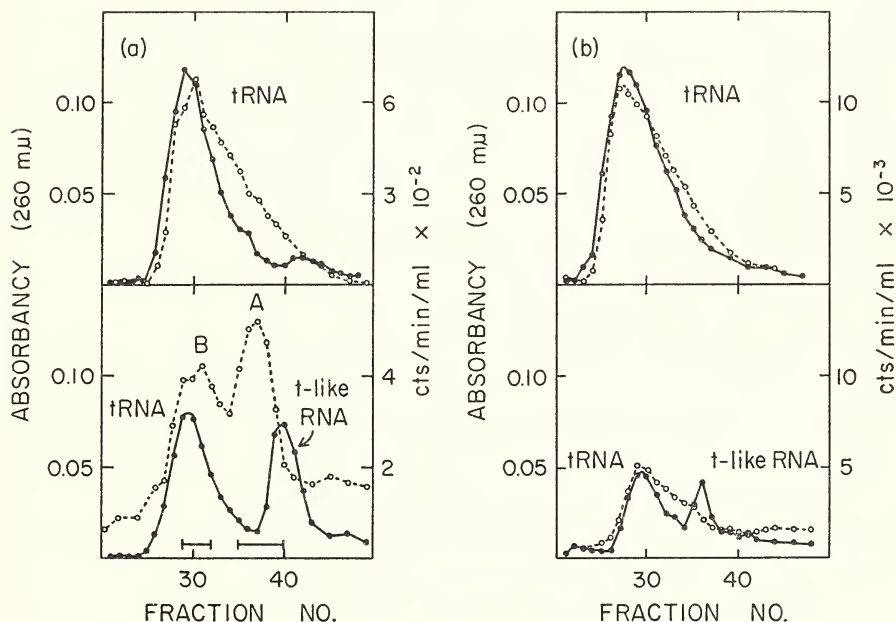
TABLE 1.—Base composition of nucleic acid fractions of *Lytechinus variegatus* embryos

Fraction	Cytosine	Guanine	Adenine	Uracil (T)
Ribosomal RNA.....	23.6	34.7	20.3	21.3
Transfer RNA.....	25.8	34.4	22.6	17.3
DNA*.....	19	19	31	31
By ^{32}P -content				
Blastula or gastrula mRNA from microsomes.....	20.6	23.8	27.7	28.0
Pluteus mRNA from microsomes....	20.6	27.8	25.7	25.8
Pluteus microsomal RNA after 2-hour chase.....	23.4	30.1	22.7	23.7
Transfer RNA peak A.....	26.0	32.6	22.4	18.9
Transfer RNA peak B.....	28.0	33.9	20.0	18.0

*By buoyant density in CsCl , $P = 1.697$.

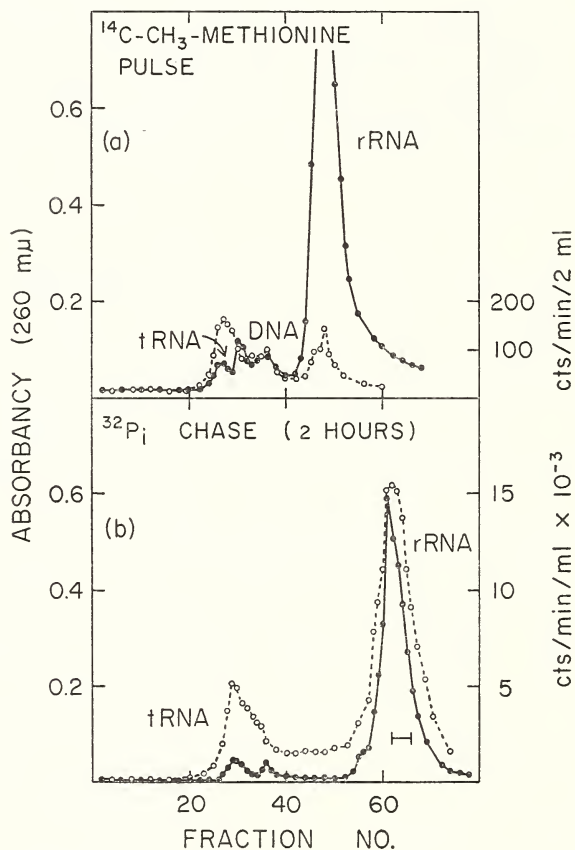
Transfer RNA

Pulse experiments with either ^{32}P i or ^{14}C -methylmethionine indicate that tRNA is first synthesized at the time of gastrulation. The synthesis of tRNA at the gastrula stage is illustrated in text-figure 5. Embryos were exposed to a 30-minute pulse of ^{32}P i; half were harvested immediately and the remainder were incubated for an additional 2 hours with excess unlabeled Pi (chase embryos). The cytoplasm was separated into a washed microsome fraction and a $105,000 \times g$ supernatant solution by centrifugation (7). The RNA was extracted from both fractions and chromatographed on methylated albumin columns. Of the total cytoplasmic tRNA, 81% was present in the $105,000 \times g$ supernatant solution and the remainder was attached to ribosomes and could not be removed by washing when the Mg^{++} concentration was maintained at 10^{-2} M. We would like to make the following points from the results presented in text-figure 5. First, the tRNA attached to ribosomes had a higher specific activity than free tRNA present in the soluble portion of the cell. Second, the ^{32}P -labeled RNA in peak A (text-fig. 5) appeared to be a precursor



TEXT-FIGURE 5.—Methylated albumin column chromatography of RNA extracted from: (a, top) the $105,000 \times g$ supernatant solution; and (a, bottom) from the microsome fraction following a 30-minute pulse of ^{32}P i ($1.0 \text{ mc}/4 \times 10^6$ embryos/40 ml of sea water) at the late gastrula stage. In (b) the same fractions are shown after the embryos had received a 2-hour chase with unlabeled Pi. Only that region of the chromatogram where tRNA and t-like RNA elute is shown. The brackets indicate those tubes which were pooled for ^{32}P -base analysis. Peak A of the microsome fraction is believed to be a precursor to tRNA. In (a, bottom) an equal amount of carrier microsomal RNA was added prior to chromatography. —●—, absorbance; —○—, radioactivity.

to tRNA. This is based on the observation that it is present only on ribosomes, and label seems to enter this peak before tRNA and disappears in the chase experiment. The base composition by ^{32}P -content is similar to that of peak B and to the over-all base composition of tRNA (table 1). Peak A synthesis corresponds exactly to tRNA synthesis during development and ^{32}P -labeled RNA of peaks A and B as well as tRNA dissociate from the ribosome when the Mg^{++} concentration is lowered to 10^{-4} M. However, the argument that peak A represents a fragment of mRNA with a base composition similar to tRNA cannot be excluded at present. Experiments are in progress to determine if the proposed precursor contains



TEXT-FIGURE 6.—(a) Methylated albumin column chromatogram of nucleic acid extracted from early pluteus nuclei after embryos had been given a 15-minute pulse of ^{14}C -methylmethionine (4×10^6 embryos/5 mc/40 ml sea water). Most of the DNA was removed from the nuclei by detergent treatment (7). (b) Methylated albumin column chromatogram of RNA extracted from the washed microsomal fraction of early plutei embryos after a 2-hour chase of the initial 30-minute pulse of $^{32}\text{P}_i$. This represented the complete chromatogram of the experiment described in text-figure 5 bottom (chase) where only the tRNA region was shown. —●—, absorbancy; ---○---, radioactivity.

base sequence homologies to tRNA and if methylated bases are present. It is of interest that the methyl-accepting RNA present on ribosomes from the aquatic fungus, *Blastocladiella emersonii* (21), elutes from methylated albumin columns at an identical position as the proposed precursor in these experiments. Finally, these studies demonstrate that no detectable synthesis of t-like RNA occurs even after a long chase and thus exclude the possibility that t-like RNA, attached to ribosomes, is a precursor to tRNA as previously postulated (22, 23).

Ribosomal RNA

Although some doubt exists as to the appearance of newly synthesized ribosomal proteins in the cytoplasm during development, more convincing evidence is available that the synthesis of ribosomal RNA does occur. This has been obtained in two ways and is illustrated in text-figure 6. Embryos exposed to ^{14}C -methylmethionine for 15 minutes at various stages of development showed incorporation of methyl groups into rRNA for the first time at the early pluteus stage. However, only the nuclear rRNA was labeled [text-fig. 6 (a)] during this short pulse. In this same experiment, there was no detectable incorporation of methyl groups into cytoplasmic rRNA. When embryos at this stage of development were given a pulse of ^{32}Pi and then allowed to develop an additional 2 hours with an excess of unlabeled Pi, labeled RNA appeared in the cytoplasm which had both chromatographic properties [text-fig. 6(b)] and base composition similarity (table 1) to those of maternal rRNA.

DISCUSSION

Although a net increase in total RNA does not occur from fertilization to the pluteus stage (24), it is clear that synthesis of each of the major species of cellular RNA is initiated. How much RNA is synthesized is difficult to estimate for several reasons, but it undoubtedly represents such a small fraction of the total RNA that it escapes detection in total estimates. One does get the impression, however, that much more emphasis is placed on mRNA and tRNA synthesis than on rRNA synthesis. Since the embryos do not feed until the late pluteus stage, and no net synthesis of protein occurs, it appears that new ribosomes are not synthesized to any great extent. Thus the question it raised as to the contribution of the nucleolus during development. Basophilic granules gradually accumulate in the nucleus, and at the gastrula stage one feels committed to state that a typical nucleolus is present, with staining and morphological characteristics of more adult type cells (14). This correlates nicely with the small amount of rRNA synthesis that does occur, but whether a nucleolus is present when tRNA is first synthesized requires closer inspection.

The synthesis of messenger RNA at the blastula stage most certainly represents the transcription of new genes, since a variety of new enzymes and adult protein can be detected shortly thereafter. The requirement for tRNA at about the same time is certainly unclear. One wonders if new species of tRNA are required for translation of the new messages. The observation that at least part of the newly synthesized tRNA appears on the ribosome prior to moving into the soluble portion of the cell and the fact that tRNA attached to ribosomes can be methylated to a much greater extent than "free" tRNA (21) provides some credence for the *modulation* hypothesis first proposed by Ames and Hartman (25) and later modified by Stent (26) and Sueoka (27). The role of tRNA in modulating or initiating gene transcription during differentiation must certainly be pursued and the sea urchin embryo may prove to be a worthy model for such investigation.

RESUMEN

Algunas de las proteínas básicas sintetizadas desde la fecundación hasta el estadio de gástrula de los embriones de erizo de mar parecen ser histonas con propiedades cromatográficas similares a las histonas del timo de ternero.

La primera síntesis detectable de proteínas básicas asociadas con los ribosomas se produce en el temprano estadio de plúteo. Con niveles específicos de isótopo y tiempo de exposición del ARN se detecta primero la síntesis en el estadio temprano de blástula y solo puede demostrarse, en este momento, síntesis de ARN mensajero. La síntesis del ARN de transferencia se inicia en el momento de la gastrulación y el ARN de transferencia recién sintetizado se asocia primariamente con la fracción ribosómica de las células. Pudo demostrarse primero, la síntesis del ARN ribosómico en el estadio temprano de plúteo. La incorporación de los grupos metilo a partir de metionina metil C^{14} en el ARN ribosómico coincide con la primera incorporación del Pi^{32} .

REFERENCES

- (1) GIUDICE, G., VITTORELLI, M. L., and MONROY, A.: Investigations on the protein metabolism during the early development of the sea urchin. *Acta Embryol Morph Exp (Palermo)* 5: 113-122, 1962.
- (2) HULTIN, T., and WESSEL, G.: Incorporation of C^{14} -labeled carbon dioxide into the proteins of developing sea urchin eggs. *Exp Cell Res* 3: 613-616, 1952.
- (3) MANGAN, J., MIKI-NOUMURA, T., and GROSS, P. R.: Protein synthesis and the mitotic apparatus. *Science* 147: 1575-1578, 1965.
- (4) SILVER, D. J., and COMB, D. G.: Unpublished results.
- (5) GLISIN, V. R., and GLISIN, M. V.: Ribonucleic acid metabolism following fertilization in sea urchin eggs. *Proc Nat Acad Sci USA* 52: 1548-1553, 1964.
- (6) BRACHET, J., DECROLY, M., FICQ, A., and QUERTIER, J.: Ribonucleic acid metabolism in unfertilized and fertilized sea-urchin eggs. *Biochim Biophys Acta* 72: 660-662, 1963.
- (7) COMB, D. G., KATZ, S., BRANDA, R., and PINZINO, C. J.: The characterization of RNA species synthesized during early development of sea urchins. *J Molec Biol* 14: 195-213, 1965.

- (8) GROSS, P. R.: The immediacy of genomic control during early development. *J Exp Zool* 157: 21-38, 1964.
- (9) GUSTAFSON, T., and HASSELBERG, I.: Studies on enzymes in the developing sea urchin eggs. *Exp Cell Res* 2: 642-672, 1951.
- (10) RANZI, S.: Proteins and antigens during the embryonic and larval development. *In* Proceedings of Symposium on Germ Cells and Development, Fond. a Baselli: Pallanza, 604-613, 1960.
- (11) WILT, F. H.: The synthesis of ribonucleic acid in sea urchin embryos. *Biochem Biophys Res Commun* 11: 447-451, 1963.
- (12) NEMER, M.: Old and new RNA in the embryogenesis of the purple sea urchin. *Proc Nat Acad Sci USA* 50: 230-235, 1963.
- (13) COMB, D. G.: Methylation of nucleic acids during sea urchin embryo development. *J Molec Biol* 11: 851-855, 1965.
- (14) COWDEN, R. R., and LEHMAN, H. E.: A cytochemical study of differentiation in early echinoid development. *Growth* 27: 185-197, 1963.
- (15) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (16) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the annucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (17) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (18) BIRNSTIEL, M. L., SIRLIN, J. L., and JACOB, J.: The nucleolus: a site of transfer ribonucleic acid synthesis. *Biochem J* 94: 10P-11P, 1965.
- (19) WALLER, J. P.: Fractionation of the ribosomal protein from *Escherichia coli*. *J Molec Biol* 10: 319-336, 1964.
- (20) MURRAY, K.: The heterogeneity of histones. *In* The Nucleohistones (Bonner, J., and Tso, P., eds.). San Francisco, Holden-Day, 1964, pp 21-35.
- (21) COMB, D. G.: The association of methyl acceptor ribonucleic acid with ribosomes. *J Biol Chem* 239: PC3597-PC3598, 1964.
- (22) COMB, D. G., and KATZ, S.: Studies on the biosynthesis and methylation of transfer RNA. *J Molec Biol* 8: 790-800, 1964.
- (23) COMB, D. G., SARKAR, N., DEVALLET, J., and PINZINO, C. J.: Properties of transfer-like RNA associated with ribosomes. *J Molec Biol* 12: 509-513, 1965.
- (24) ELSON D., GUSTAFSON, T., and CHARGAFF, E.: The nucleic acids of the sea urchin during embryonic development. *J Biol Chem* 209: 285-294, 1954.
- (25) AMES, B. N., and HARTMAN, P.: The histidine operon. *In* Synthesis and Structure of Macromolecules. Sympos Quant Biol 28: 1963, pp 349-356.
- (26) STENT, G. S.: The operon: On its third anniversary. *Science* 144: 816-820, 1964.
- (27) SUEOKA, N., and KANO-SUEOKA, T.: A specific modification of leucyl-sRNA of *Escherichia coli* after phage T2 infection. *Proc Nat Acad Sci USA* 52: 1535-1540, 1964.

DISCUSSION

Sirlin: When you say that the tRNA is methylated while attached to the ribosome, are you differentiating here between nuclear and cytoplasmic ribosomes or do you mean ribosomes in general?

Comb: With the sea urchin system, after methionine pulse and examination of the methylated bases, one finds that the specific activity of tRNA in the nucleus is much higher than in the cytoplasm. What I am proposing is that newly synthesized tRNA is methylated in the nucleus and enters the cytoplasm attached to a ribosome. Methylation occurring in the cytoplasm may represent the methylation of the bind-

ing site to the ribosome and this is possibly the TpYpCpGp sequence described by Zamur, Holley, and Marquisee (J Biol Chem 240:1267-2173, 1965).

Sirlin: So there are two different methylations?

Comb: Yes. We believe that some methylation occurs in the cytoplasm.

Brown: Do you mean that a molecule which is further methylated on the ribosome can be used preferentially over the remainder of the tRNA?

Comb: Yes, this is probably true, although we have no direct evidence for this.

Taylor: I understood you to say in the first part of your talk that this "t-like" RNA was firmly attached to some structure so that it did not come off easily. Was it attached to rRNA?

Comb: Yes, it is attached to the 50S ribosome subunit, not to the 30S.

Taylor: It is not removed by lowering the concentration of magnesium?

Comb: That is right. In fact, we originally thought that t-like RNA attached to the 50S subunit might be a precursor of tRNA since it contained no detectable methylated bases and very low amounts of pseudouridine (0.42% of the total bases). It looked like tRNA by base composition and it was more active than tRNA in accepting methyl groups. However, when dialyzing the ribosomes against dilute Mg^{++} , the methyl-accepting activity dissociates with the tRNA while the t-like RNA remains attached to the 50S subunit.

Nucleolar RNA Synthesis During Mitotic and Meiotic Prophase^{1, 2}

NIRMAL K. DAS and MAX ALFERT,³ *Department of Zoology, University of California, Berkeley, California*

SUMMARY

Autoradiographic studies, using a pulse-labeling procedure, show that the nucleolar apparatus is a very active center of RNA synthesis during early mitotic prophase in *Allium* and *Nigella* root meristems and during meiotic prophase in *Urechis* oocytes. In contrast to these, the nucleolar apparatus produces little or no RNA during meiotic prophase in

corn anthers and in locust testis; in male reproductive cells of *Urechis*, the nucleolar apparatus seems to be lost prior to meiosis. This suggests a switching off of nucleolar genes, which are presumed to be involved in ribosomal RNA synthesis, prior to or during male meiosis.—*Nat Cancer Inst Monogr* 23: 337–351, 1966.

THAT THE nucleolar apparatus is an active site of RNA biosynthesis has been well documented in a number of studies, *e.g.*, (1). It has been shown recently that genes involved in the production of ribosomal RNA are clustered in the chromosome regions associated with the nucleolus and that ribosomes are made in the nucleolus (2–4). In this paper we will review the results of our autoradiographic studies that support the idea of an independent RNA synthesis in the nucleolar apparatus. Some of the results presented here have been published earlier (5–8) and, therefore, they will be discussed briefly. Other unpublished results will be dealt with more extensively.

Prophase cells are selected for a quantitative analysis of RNA synthesis, by use of pulse-labeling procedures, in the nucleolar apparatus versus the rest of the chromatin to answer the following questions: Is the rate of synthesis in the nucleolar apparatus during early mitotic prophase affected when a decrease in the rate of synthesis in the condensing chromatin takes place? Does the rate of RNA synthesis in the nucleolar

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² These studies were supported by grants from the National Science Foundation and the U.S. Public Health Service.

³ We wish to thank Mrs. E. P. Siegel for technical assistance.

apparatus differ from that of the rest of the nucleus as oocytes mature during meiotic prophase? Is there any difference in RNA synthetic activity of the nucleolar apparatus during female and male meiosis?

The experimental materials were root-tip cells of onion (*Allium cepa*) and fennel flower (*Nigella arvensis*), corn (*Zea mays*) microsporocytes, unfertilized eggs of *Urechis caupo* (a marine echiuroid worm), developing oocytes and male reproductive cells of *Urechis*, and locust (*Schistocerca gregaria*) spermatocytes. The materials, except *Urechis* cells, were incubated at room temperature (ca. 24 C) in media with added H³-cytidine or H³-uridine (100–250 μ c/ml; specific activity 1.25–8.1 c/mm). The incorporation studies with *Urechis* cells were conducted at 22 C or below. Samples were fixed in acetic alcohol or in 10% neutral formalin. Autoradiographs of squashed preparations or 5 μ paraffin sections were made by use of a liquid emulsion (NTB2) or a stripping film (AR10). That the tritiated precursors were mainly incorporated into RNA was checked by digesting slides with ribonuclease prior to coating with emulsion.

RESULTS AND DISCUSSION

In onion and fennel-flower root tips, exposed to H³-cytidine for 6 minutes, the rate of RNA synthesis per unit area in the chromatin fraction decreases significantly from interphase to early prophase, while the rate in the nucleolar apparatus remains normal (table 1). This shows that RNA synthesis in the nucleolar apparatus is independent of the remaining chromatin. This conclusion is further borne out by the fact that RNA synthesis in the late telophase cell resumes predominantly in the nucleolar bodies (figs. 1A and 1B).

That RNA synthesis in the nucleolar apparatus is not dependent on the rest of the nucleus can also be demonstrated in *Urechis* oocytes at various stages of growth during meiotic prophase. The coelomic fluid of female *Urechis* containing oocytes of various sizes was incubated *in vitro* for 30 minutes with H³-cytidine. Table 2 presents the data on silver grain counts over nucleus (minus nucleolus) and nucleolar fractions. On the basis of grains per section, the smallest oocytes, which are presumably in premeiotic stages and which usually appear in clusters in the coelomic fluid, seem to be least active in RNA synthesis. These oocytes contain a relatively small nucleolus and a number of "prochromosomes." As the oocytes become larger (classes I–IV), the average grain number per nucleolar section increases. Such an increase is less obvious in the nuclear sections. On a per unit area basis, the rate of RNA synthesis in the nucleolar apparatus does not change much throughout the different stages of oocyte growth, while the rate of synthesis in the rest of the nucleus decreases gradually (table 2).

When these data are presented in terms of total nuclear and nucleolar radioactivity, it becomes apparent that the increase in the nucleolar volume

TABLE 1.—Incorporation of H³-cytidine for 6 minutes in root-tip cells of *Allium* and *Nigella*

Nuclear stages	Grains per unit area × 10					
	<i>Allium cepa</i>			<i>Nigella arvensis</i>		
	Whole nucleus	Chromatin alone	Nucleolus alone	Whole nucleus	Chromatin alone	Nucleolus alone
Interphases.....	5.6 ± 0.34	3.8 ± 0.22	14.0 ± 1.09	6.6 ± 0.42	3.7 ± 0.37	16.3 ± 1.24
Number of nuclei.....	40			18		
Early prophase.....	3.8 ± 0.27	2.3 ± 0.17	12.6 ± 0.82	5.3 ± 0.16	2.7 ± 0.19	16.8 ± 1.24
Number of nuclei.....	28			33		

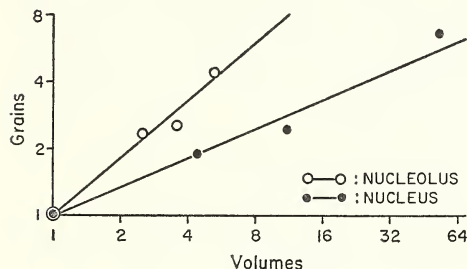
Reproduced from an earlier paper in *Science* (5), with the kind permission of the publisher. Secondary roots of *Allium* were exposed to 100 µc/ml and the primary roots of *Nigella* to 50 µc/ml of H³-cytidine. There was no incorporation of H³-cytidine from late prophase through early telophase cells. Exposure times of film were 1 week for squashed preparations of *Nigella* root tip and 2 weeks for 5 µ thick sections of *Allium*. Cells were stained with methyl green-pyronine through the processed film.

TABLE 2.—Incorporation of H³-cytidine (150 μ c/ml) for 30 minutes in the developing oocytes of *Urechis*

Cell stage	Number of sections	Nucleus (minus nucleolus)			Nucleolus		
		Average diameter (μ)	Average grains/section	Average grains/100 μ^2	Average diameter (μ)	Average grains/section	Average grains/100 μ^2
Premeiotic interphase.....	13	5-8 (6.6)	6.1 \pm 0.7	14.0 \pm 1.5	2.9	3.7 \pm 0.6	44.4 \pm 6.8
Meiotic prophase	29	6-12 (9.8)	10.4 \pm 0.8	11.9 \pm 1.1	5.4	20.2 \pm 2.2	70.7 \pm 7.5
I.....	32	13-19 (16.2)	12.3 \pm 1.0	4.9 \pm 0.4	7.5	34.9 \pm 3.4	61.3 \pm 5.9
II.....	26	20-26 (22.2)	10.8 \pm 1.1	2.3 \pm 0.2	8.4	33.7 \pm 3.9	49.5 \pm 5.9
III.....	32	≥ 27 (36.9)	17.5 \pm 1.7	1.3 \pm 0.1	9.5	49.4 \pm 4.7	55.9 \pm 5.5
IV.....							

No cytoplasmic labeling was seen during this time. Grains per section were corrected for the background. Autoradiographs of sections (5 μ) were exposed for 5½ hours. Sections were stained with Harris' hematoxylin through the processed emulsion.

approximately equals the increase in the total nucleolar radioactivity (text-fig. 1). Such a direct proportionality does not exist in the nuclear fraction (text-fig. 1). The oocytes of other materials were also found to contain a nucleolar apparatus very active in RNA synthesis (9-12). In maturing sea urchin oocytes, a rapid increase in the azure B-stained nucleolar RNA content has also been observed by Swift *et al.* (13). Ruthmann (14) reported that, in developing oocytes of *Ophryotrocha*, RNA syntheses in the nuclear and nucleolar fraction are independent of each other.



TEXT-FIGURE 1.—The relationship between volumes and total radioactivity in different classes (I-IV; table 2) of oocytes. The average volumes and grains per compartment of the class I are used as 1.

The unfertilized eggs of *Urechis*, which are stored at diakinesis in sacs, also possess a synthetically active nucleolar apparatus. Figure 2 shows that the eggs become labeled in the nucleus, especially in the nucleolus after a 15-minute exposure to H^3 -uridine at 14-17 C. When the time of exposure to H^3 -uridine is increased from 15 to 60 minutes, labeled RNA accumulates at a faster rate in the nucleolar apparatus than in the rest of the nucleus (figs. 2 and 3); the average grain ratios, on a per unit area basis, of the nucleolus to the rest of the nucleus were 12 and 28 for the 15- and 60-minute samples, respectively.

The unfertilized eggs, as well as oocytes of various sizes, contain numerous small nucleolar-like bodies or accessory nucleoli in addition to a large main nucleolus. Possibly, these bodies accumulate because of the slow rate of movement of nuclear RNA into the cytoplasm. Aspects of nuclear and cytoplasmic labeling of eggs are illustrated in figures 4, 5, and 6. Some somatic cells of *Urechis* show heavy cytoplasmic label, in contrast to eggs, after 17½ hours' incubation at 22 C in the chase medium following 1-hour initial labeling with H^3 -uridine. When the unfertilized eggs, labeled initially for 1 hour, are incubated for 17½ hours at 4 C, instead of 22 C, little or no increase in the nuclear or nucleolar labeling occurs; the cytoplasm also remains completely unlabeled (figs. 4, 5, and 6). Thus, further synthesis and transport are blocked. The low temperature also results in accumulation of accessory nucleoli as large as, and sometimes larger than, the main nucleolus. About 60% of the unfertilized eggs incubated at low temperature for a period of 17 to 26 hours contain these large accessory nucleoli in addition to main nucleoli. Staining reactions of the accessory nucleoli are shown in figures 7A through 9B (15, 16).

The accessory nucleoli are not formed from materials made during incubation in the cold, since eggs incubated at low temperature (4 C) for 20 hours with added 100–150 $\mu\text{C}/\text{ml}$ of H^3 -cytidine or H^3 -uridine do not show labeled accessory nucleoli, even though the nucleus and the main nucleolus become slightly labeled during this time. When the eggs were labeled with H^3 -cytidine for 21 to 42 hours at 22 C and then incubated at 4 C in the chase medium for 24 hours, the accessory nucleoli became labeled (figs. 10A and 10B). Similar results were also obtained with H^3 -phenylalanine (figs. 11A and 11B). Thus, the accessory nucleoli are formed, possibly by fusion, from the ribonucleoprotein bodies made before the eggs were transferred to low temperature. In this connection, Beermann (17) observed an accumulation of such bodies, which he called "Neben-nukleolen," around the Balbiani ring of *Chironomus* giant chromosomes when the larvae were kept at 5 C and then brought back to room temperature for an hour; these nucleoli disappeared within the next 8 hours at room temperature. In *Urechis* eggs, the accessory nucleoli induced by low temperature also break down to smaller bodies when the eggs are brought back to 22 C for 1 hour or so. These nucleoli are inactive with respect to RNA synthesis, perhaps due to their lack of association with chromosome regions.

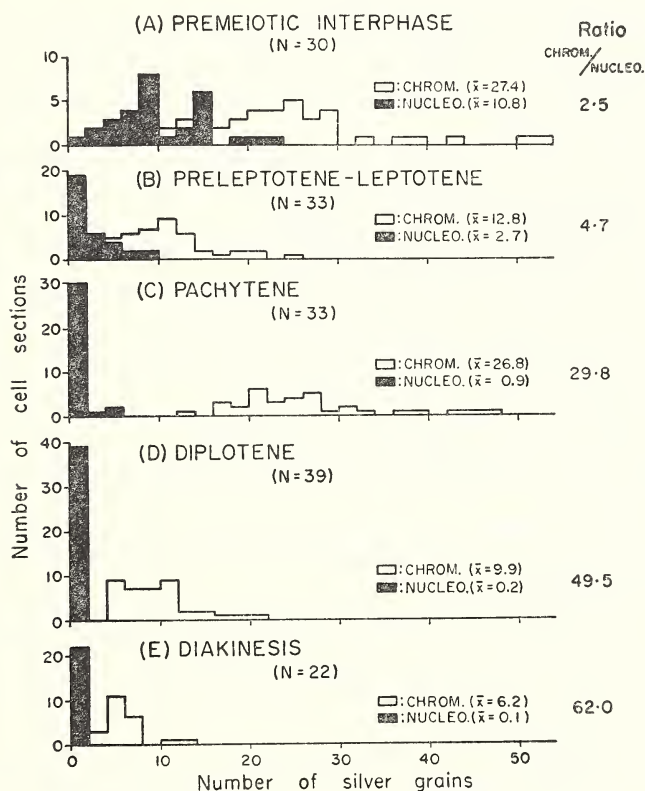
Recent biochemical studies (2–4) have provided evidence that the nucleolar apparatus is actively engaged in ribosomal RNA and ribosome synthesis. On the basis of such evidence, one would expect the nucleolar apparatus to be highly active in dividing tissues such as root meristems. The oocytes must also be endowed with an active nucleolar apparatus to produce ribosomes necessary for protein synthesis during early embryogenesis (18). However, the maintenance of a highly active nucleolar apparatus during male gametogenesis seems unnecessary, since the sperm contributes no appreciable amount of cytoplasm to the growth of the embryo. Some of our studies on RNA synthesis in male reproductive cells and male gametophytes are discussed below and show that loss or physiological inactivation of the nucleolar apparatus appears to characterize male meiosis.

Unlike the primary oocytes, the male reproductive cells of *Urechis* apparently lose the nucleolar apparatus prior to meiosis. The male reproductive cells are present in clusters in the coelomic fluid. The youngest of these cells, which are diffusely stained with Feulgen, contain a nucleolus (fig. 12) active in RNA synthesis. Gradually their nuclei become condensed (fig. 13) and undergo DNA synthesis leading to an accumulation of clusters of cells at the G_2 stage. The nucleolar apparatus is no longer detectable in these cells (fig. 13) and the rate of RNA synthesis also decreases.

The autoradiographic studies on RNA synthesis during spermatogenesis in the locust show that RNA synthesis in the nucleolar apparatus of the mid-meiotic prophase is inconspicuous [see also (19)]; most of the RNA in these cells is made by the autosomes (fig. 14). RNA synthesis in autosomes

continues at late prophase when nucleoli are not seen (fig. 15). Swift *et al.* (13) also observed that, in grasshopper spermatocytes, the azure B-bound nucleolar RNA content dropped rapidly as the meiotic prophase progresses.

In contrast to the male reproductive cells of *Urechis* and the meiotic prophase cells of locust, corn microsporocytes at prophase I maintain a prominent nucleolar apparatus. Our study, in which corn anthers were labeled for 2 hours with H^3 -cytidine or H^3 -uridine, shows that, though the nucleolar apparatus of the premeiotic interphase is active in RNA synthesis, a rapid inactivation of RNA synthesis in the nucleolar apparatus occurs as the meiotic prophase progresses; RNA synthesis in these cells is confined only to chromosomes, even though they contain a persistent highly basophilic nucleolus (figs. 16, 17, and 18; text-fig. 2).



TEXT-FIGURE 2.—Distribution of silver grains over chromosomal and nucleolar fraction from cells at premeiotic interphase and various stages of meiotic prophase I. N = number of cell sections ($5\ \mu$); \bar{x} = mean number of grains; Chrom. = chromosome; Nucleo. = nucleolus.

Reproduced from an earlier paper in *Experimental Cell Research* (8), with the kind permission of the publisher.

RESUMEN

Los estudios autoradiográficos que emplean el procedimiento de marcación por pulsos muestran que el aparato nuclear es un centro muy activo de síntesis del ARN durante la profase temprana mitótica en los meristemas radiculares de *Allium* y de *Nigella* y durante la profase meiótica de los ovocitos de *Urechis*.

En contraste con éstos, el aparato nucleolar produce poco o ningún ARN durante la profase meiótica en anteras de maíz y en testículos de langosta; en las células reproductoras de *Urechis* el aparato nucleolar parece perderse antes de la meiosis. Esto sugiere una interrupción de los genes nucleolares que se presume que están involucrados en la síntesis del ARN ribosómico antes o durante la meiosis masculina.

REFERENCES

- (1) BUSCH, H., BYVOET, P., and SMETANA, K.: The nucleolus of the cancer cell: a review. *Cancer Res* 23: 313-339, 1963.
- (2) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosomes. *Biochim Biophys Acta* 76: 454-462, 1963.
- (3) MCCONKEY, E. H., and HOPKINS, J. W.: The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 51: 1197-1204, 1964.
- (4) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (5) DAS, N. K.: Chromosomal and nucleolar RNA synthesis in root tips during mitosis. *Science* 140: 1231-1233, 1963.
- (6) DAS, N. K., SIEGEL, E. P., and ALFERT, M.: Synthetic activities during spermatogenesis in the locust. *J Cell Biol* 25: 387-395, 1965.
- (7) DAS, N. K., LUYKX, P., and ALFERT, M.: The nucleolus and RNA metabolism in *Urechis* eggs. *Develop Biol* 12: 72-78, 1965.
- (8) DAS, N. K.: Inactivation of the nucleolar apparatus during meiotic prophase in corn anthers. *Exp Cell Res* 40: 360-364, 1965.
- (9) VINCENT, W. S.: Structure and chemistry of nucleoli. *Int Rev Cytol* 4: 269-298, 1955.
- (10) FICQ, A.: Étude autoradiographique du métabolisme des protéines et des acides nucléiques au cours de l'oogénèse chez les batraciens. *Exp Cell Res* 9: 286-293, 1955.
- (11) ———: Étude autoradiographique du métabolisme de l'oocyte d'*Asterias rubens* au cours de la croissance. *Arch Biol (Liege)* 66: 509-524, 1955.
- (12) EDSTRÖM, J.-E., GRAMPP, W., and SCHOR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. *J Biophys Biochem Cytol* 11: 549-557, 1961.
- (13) SWIFT, H., REBHUN, L., RASCH, E., and WOODARD, J.: The cytology of nuclear RNA. In *Cellular Mechanisms in Differentiation and Growth* (Rudnick, D., ed.). New Jersey, Princeton University Press, 1956, pp 45-59.
- (14) RUTHMANN, A.: Zellwachstum und RNA-synthese im Ei-nährzellverband von *Ophryotrocha puerilis*. *Z Zellforschung* 63: 816-829, 1964.
- (15) FLAX, M. H., and HIMES, M. H.: Microspectrophotometric analysis of meta-chromatic staining of nucleic acids. *Physiol Zool* 25: 297-311, 1952.
- (16) DAS, N. K.: Demonstration of a non-RNA nucleolar fraction by silver staining. *Exp Cell Res* 26: 428-431, 1962.

- (17) BEERMANN, W.: Chromomerenkonstanz und spezifische Modifikationen der Chromosomenstruktur in der Entwicklung und Organdifferenzierung von *Chironomus tentans*. Chromosoma 5: 139-198, 1952.
- (18) DAVIDSON, E. H., ALLFREY, V. G., and MIRSKY, A. E.: On the RNA synthesized during the lampbrush phase of amphibian oogenesis. Proc Nat Acad Sci USA 52: 501-508, 1964.
- (19) HENDERSON, S. A.: RNA synthesis during male meiosis and spermiogenesis. Chromosoma 15: 345-366, 1964.

PLATE 44

All cells shown in this plate were fixed in acetic alcohol except those cells shown in figures 8A and 8B which were fixed in 10% neutral formalin.

FIGURES 1A AND 1B.—Autoradiographs of a late telophase cell of *Nigella* root tip labeled with H^3 -cytidine (50 μ c/ml) for 6 minutes (fig. 1A); the same cell was stained with silver (fig. 1B) to show nucleolar bodies over which grains are mainly localized. Chromosomes were stained with methyl green. Film exposure time: 1 week. \times 1,380

FIGURES 2 AND 3.—Autoradiographs of sections (5 μ) of unfertilized *Urechis* eggs labeled at 14 to 17 C with H^3 -uridine (150 μ c/ml) for 15 and 60 minutes, respectively. Note the marked labeling of the nucleolus. Cells were stained with hematoxylin. Emulsion exposure time: 17 days. \times 650

FIGURES 4, 5, AND 6.—Autoradiographs of sections (5 μ) of unfertilized *Urechis* eggs labeled at 22 C with H^3 -uridine (100 μ c/ml) for 1 hour (fig. 4) and then incubated for 17½ hours at 22 C (fig. 5) and at 4 C (fig. 6). Cells were stained with hematoxylin. Emulsion exposure time: 12 days. \times 650

FIGURES 7A, 7B, 8A, 8B, 9A, AND 9B.—Azure B (figs. 7A and 7B), acid-fast green (figs. 8A and 8B), and silver (figs. 9A and 9B) staining of 10 μ sections of unfertilized *Urechis* eggs incubated for 20 hours at 22 C (figs. 7A, 8A, and 9A) and at 4 C (figs. 7B, 8B, and 9B). MN = main nucleolus; AN = accessory nucleolus. Figures 7A through 8B: \times 900; figures 9A and 9B: \times 600

Figures 1A, 1B, 2, and 3 are reproduced from earlier papers in *Science* and *Developmental Biology* (5, 7), with the kind permission of the publishers.

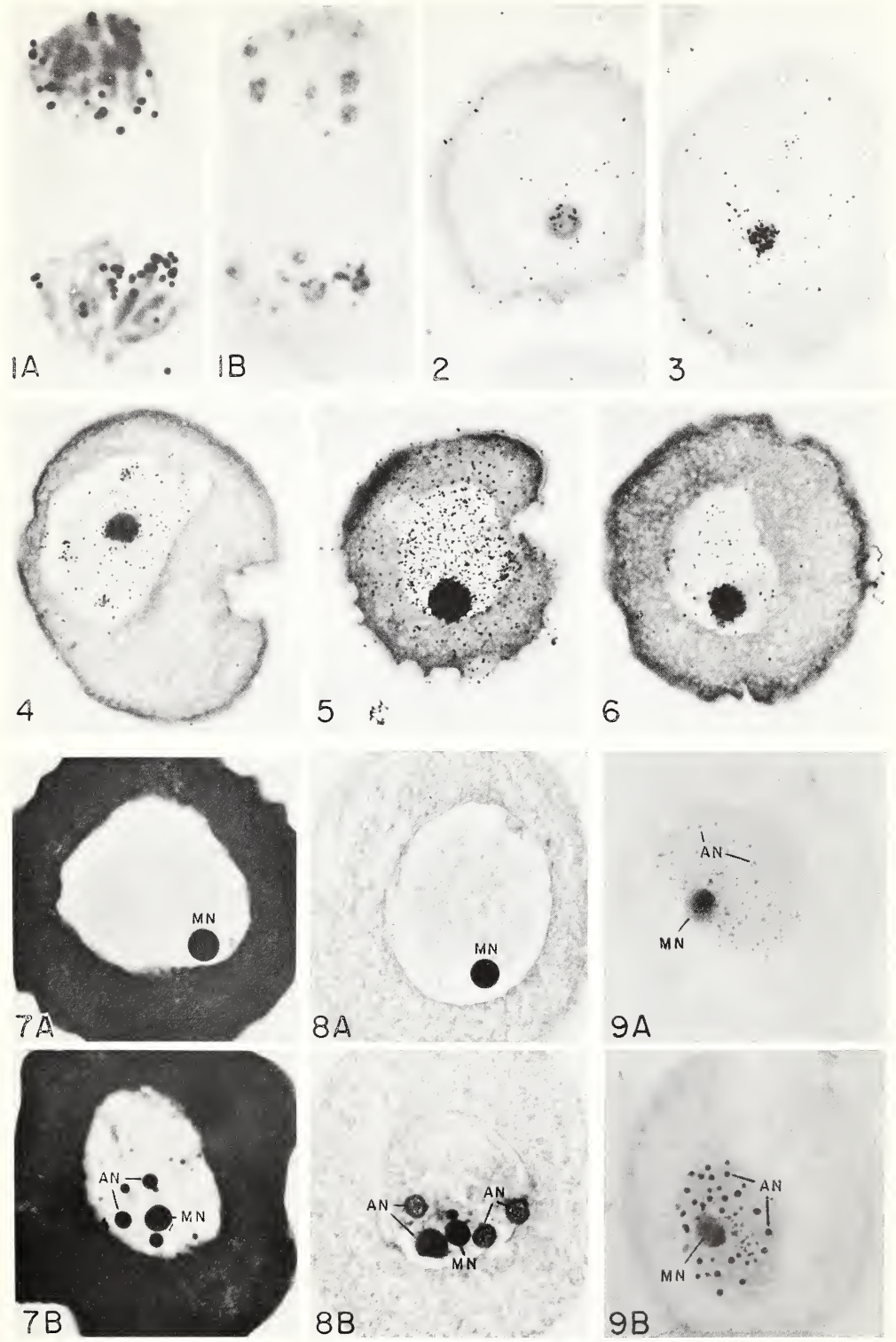


PLATE 45

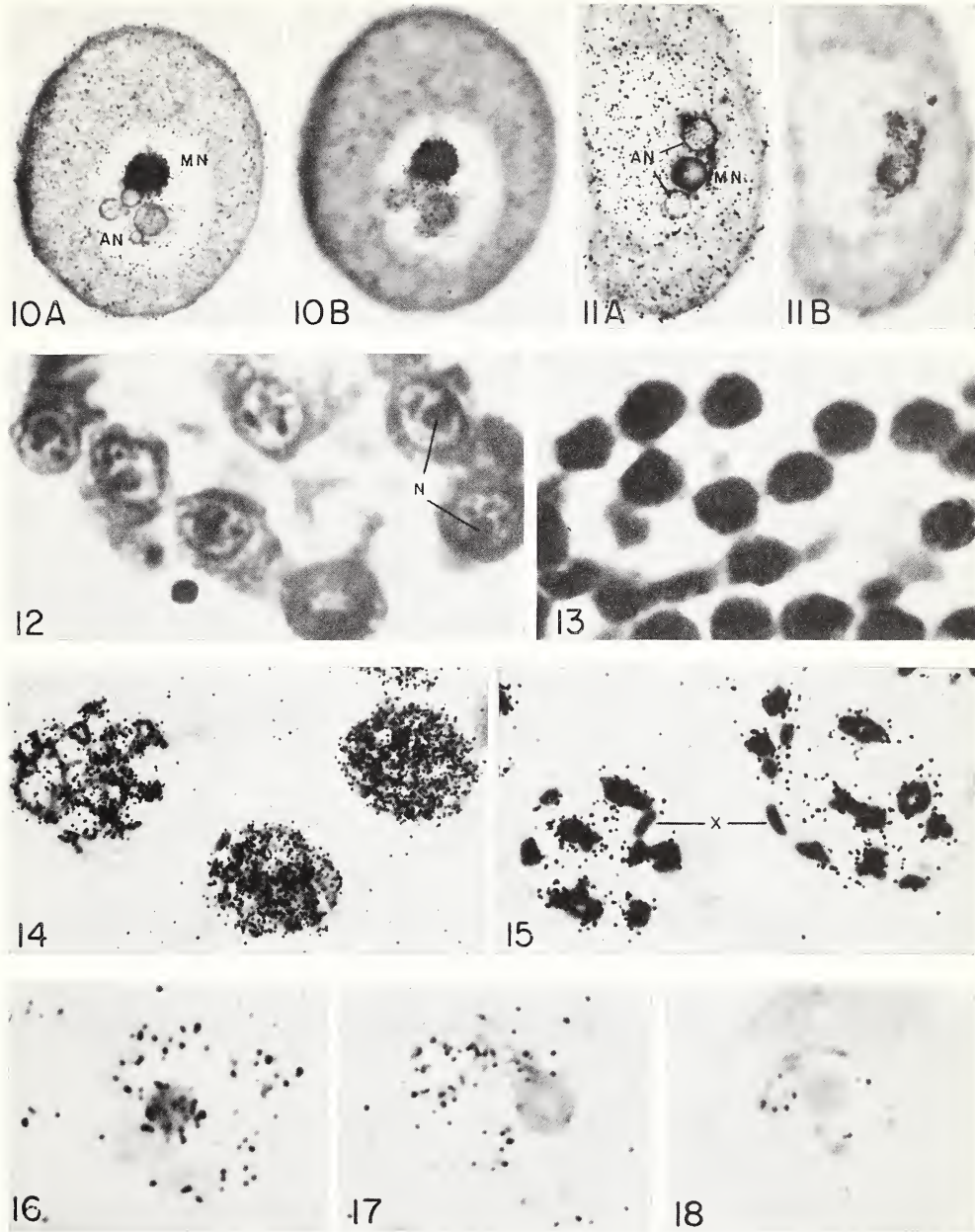
All cells shown in this plate, except those shown in figures 10A, 10B, 11A, and 11B, were fixed in acetic alcohol: cells shown in figures 10A, 10B, 11A, and 11B were fixed in 10% neutral formalin.

FIGURES 10A, 10B, 11A, AND 11B.—Autoradiographs of sections ($5\ \mu$) of unfertilized *Urchis* eggs labeled at 22 C with H^3 -cytidine (100 $\mu\text{c}/\text{ml}$) for 21 hours (figs. 10A and 10B) and with H^3 -phenylalanine (25 $\mu\text{c}/\text{ml}$) for 42 hours (figs. 11A and 11B) and then incubated for 24 hours at 4 C in the chase medium. A and B are two photographs of the same cell, focussed on the cell (A) and on the grains over nucleoli (B). MN = main nucleolus; AN = accessory nucleolus. Cells were stained with hematoxylin. Emulsion exposure time: 6 days (fig. 10) and 4 days (fig. 11). $\times 650$

FIGURES 12 AND 13.—Azure B-stained sections ($3\ \mu$) of male reproductive cells of *Urchis* showing the presence of nucleolus (N) in younger cells (fig. 12) and its absence when nuclei become condensed (fig. 13). $\times 1,250$

FIGURES 14 AND 15.—Autoradiographs of locust spermatocytes, labeled with H^3 -cytidine (250 $\mu\text{c}/\text{ml}$) for 2 hours, at pachytene-diplotene (fig. 14) and at diakinesis (fig. 15). Note absence of conspicuous nucleolar labeling and the absence of labeling of the X chromosome in diakinesis (X); label is confined to the autosomes. Cells were stained with hematoxylin. Emulsion exposure time: 5 days (fig. 14) and 2 days (fig. 15). $\times 700$

FIGURES 16, 17, AND 18.—Autoradiographs of sections ($5\ \mu$) of corn microsporocytes, labeled with H^3 -cytidine (200–250 $\mu\text{c}/\text{ml}$) for 2 hours, at premeiotic interphase (fig. 16), pachytene (fig. 17), and at diakinesis (fig. 18). Note only the nucleolus of the premeiotic interphase cell is heavily labeled; chromosomes in all cells are labeled. Cells were stained with hematoxylin. Emulsion exposure time: 8 weeks. $\times 1,550$



Figures 14, 15, 16, 17, and 18 are reproduced from earlier papers in *Journal of Cell Biology* and *Experimental Cell Research* (6, 8), with the kind permission of the publishers.

DISCUSSION

Pavan: I would like once more to emphasize the question of what name to give to the RNA-containing bodies found in the nucleus. Dr. Das told us about nucleolar-like bodies. I used to call them "micronucleoli," and then I decided to call them "RNA bodies," and then Dr. Barr talked about "blobs." And I insist that we should have a name for this type of structure because we probably are talking about similar things that exist in the nucleus but which are not related to or not produced directly by the nucleolar organizer region. I do agree that we should not invent too many names, but to facilitate the lives of the students of Biology, I propose to the nomenclature committee "nucleoloides" as an easy and useful name.

Das: I would like to add further that the small nucleolar-like bodies, present in unfertilized eggs of *Urechis*, contain ribonucleoproteins, since they stain with azure B, acid-fast green, or silver. These bodies (or blobs, as called by Dr. Barr) are also seen at interphase in *Urechis* embryos during early cleavage stages, particularly when the sensitive silver-staining technique is used [(16), this paper; Tandler, Exp Cell Res 17: 560-564, 1959]. In my opinion, we should not worry about the nomenclature of these bodies, since we do not know anything about their function at the present time. However, the silver-stainable structures detected in some X-radiation-induced micronuclei (Das, J Cell Biol 15: 121-130, 1962) and in late telophase cells [(5), this paper] in root meristems seem to be truly nucleolar in nature as far as their ability to incorporate labeled RNA precursors is concerned.

Sirlin: There are two particular points made at this Symposium that strike me as inconsistent. The first one concerns the prenucleolar bodies. It seemed to me that it was pretty well accepted until this Symposium (truth is not fixed forever, but there didn't seem to be any great arguments about this) that these prenucleolar bodies, at least some of them, form the nucleolus. Das, himself, has been one of the most recent ones to provide evidence in this respect. Now, suddenly we have found people in this Symposium just coming along and saying, "I didn't say what I said two months ago," without evidence to back up their denial. I think this is not a question of nomenclature; I think it is a question of convincing some laboratories to run some time-lapse cinematography to see what happens in a well-defined cell with these prenucleolar bodies. The second point concerns the "micronucleoli" that Pavan has observed in the polytene nuclei. Again, it is not so much a question of nomenclature as simply a question of unifying our opinion as to what they are and under what conditions we see them, and then whether we can call them nucleoli. To me, some of them are obviously scattered chromosomal products, not nucleoli.

Tandler: As Dr. Das has said, the silver-staining technique is specific and sensitive. On some of the pictures I projected, one can see these structures, call them nucleolar-like or prenucleolar bodies or whatever you wish, with the silver technique. Now, concerning their origin, the nucleolus is about 90% protein. In the interphase nucleus, when there is a nucleolus at the organizer and proteins are being synthesized in this nucleolar body, the messenger RNA which codes for these proteins may not, or need not, be localized at the DNA organizer. If this is so, one might expect that, under certain circumstances, these nucleolar proteins would accumulate at several loci within the nucleus, *c.g.*, when the organizer is not present, as in the anucleolate mutants, aberrant maize pollen tetrads, and Dr. Das' micronuclei, or even before a nucleolus forms after mitosis. I think this is what actually happens. It is interesting also that these bodies usually have ribonuclease-removable RNA, and that their ultrastructure, as Dr. Swift has shown for maize pollen and as has been shown in the anucleolate *Xenopus* mutant too, seems to be similar to what is called the fibrillar or homogeneous part of the definitive nucleoli.

Swift: Dr. Tandler, it seems to me less important what you call these structures than what you don't call them. I don't think they should be called nucleoli until one can show that they substitute effectively for the true nucleolus formed at the organizer.

Dr. Das, your observation of rapid RNA precursor incorporation into all the chromosomes of the grasshopper meiotic prophase, except for the X chromosome, is very interesting. In this connection I would like to mention a finding Dr. Saez made when he was working with Pollister at Columbia in 1952. In his spectrophotometric studies on grasshopper testes stained with azure B, he showed that the autosomes possessed marked metachromatic staining, characteristic of RNA, while the X remained orthochromatic, showing that it primarily contained DNA and no detectable RNA. Your results are certainly in agreement with these findings.

Das: I am sorry. I should have mentioned the cytophotometric study of Dr. Saez (Riv Istoch Norm Pat 1 : 485, 1955).

Amino Acid Incorporation Patterns During the Cell Cycle of Synchronized Human Tumor Cells^{1,2}

FREDERICK H. KASTEN³ and FREDY F. STRASSER,
*Pasadena Foundation for Medical Research, Pasadena,
California 91101; Department of Anatomy, University
of Southern California School of Medicine; Department
of Pathology, Loma Linda University School of Medicine.
Loma Linda, California 92354.*

SUMMARY

Cultured CMP epithelial human tumor cells were synchronized in the cell cycle using a double-sequential blockage with excess thymidine. A high level of synchrony was achieved according to various criteria and yielded mitotic bursts as high as 70%. Autoradiographic techniques were employed in conjunction with tritiated arginine, lysine, leucine, tryptophane, and glycine to detect intracellular incorporation patterns. Arginine is incorporated into chromatin and nucleoli during the S phase and into chromatin during the G2 phase. The G1 phase is characterized by preferential or selective incorporation of all 5 amino

acids into nucleoli. This usually occurs a few hours after completion of mitosis. It is suggested that this G1 burst in uptake is due particularly to synthesis of arginine-rich histones in the nucleolus. There is 1.5–3.0 times more lysine incorporated into nuclei (chromatin and nucleolus) than into cytoplasm during the cell cycle. The terminal S period is characterized by a sudden increase in chromatin- and nucleolar-associated lysine. These lysine-rich proteins may be significant in relation to complexing of histone with DNA or in triggering mitosis.—*Nat Cancer Inst Monogr* 23: 353–368, 1966.

ONE OF the major goals in mammalian cell biology is the elucidation of nucleoprotein synthetic patterns and their control mechanisms. Since chromosomal DNA replication occurs in definite sequential steps (1, 2) during a relatively constant portion of the cell cycle (3), it is apparent that

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported in part by U.S. Public Health Service research grants CA-07991-01 and CA-07991-02 from the National Cancer Institute, and NB-03113-05, from the National Institute of Neurological Diseases and Blindness, and the Milheim Foundation for Cancer Research. The contributions of C. George Lefebvre, Stephen Anderson, Tom Gardner, and Marian Turner are gratefully acknowledged.

³ The author's participation in this Symposium was made possible with travel funds made available from the U.S. Public Health Service research grant NB-03113-06, from the National Institute of Neurological Diseases and Blindness, and the Pasadena Foundation for Medical Research.

this orderliness is guided by an intricate regulatory system. A number of lines of evidence implicate histones in genetic regulation (4), but it is unclear whether they play a significant role in initiating DNA replication and in controlling the stepwise pattern of synthesis along the length of each chromosome and in the entire genetic complement. The fact that one or more histone fractions are associated into a nucleoprotein chromosomal complex requires that these histones likewise undergo sequential replication. The presence of extrachromosomal histones and DNA in the nucleolus adds further complications, since the functions subserved by the nucleolus undoubtedly require other distinctive patterns of incorporation and biosynthesis in this organelle.

Randomly growing cell populations obviously present difficulties in dealing with these problems because of the exact nature of nucleoprotein synthetic patterns during the cell cycle. The utilization of synchronized cultures circumvents this difficulty and allows precise autoradiographic analyses of incorporation patterns of labeled precursors into nucleic acids and proteins. Detailed observations of nucleic acid synthetic patterns were presented at the 1965 meeting of the American Society for Cell Biology and are being published elsewhere (5). Briefly, we found that RNA synthesis is primed for the most part by intranucleolar DNA and to a lesser extent by chromosomal DNA. These principal template DNA fractions are themselves replicated during the early S phase of the cell cycle. Shortly afterward in the mid-S phase, a second nucleolar DNA fraction is synthesized. It differs from the first fraction in its time relations and in the fact that nucleolar RNA synthesis is not simultaneously repressed.

During this Symposium, a film was presented detailing by phase-contrast, time-lapse cinematography the cytology of living cells during selected phases of the synchronized cell cycle. In addition, the film displayed electron micrographs derived from synchronized cultures. In this report we summarize the incorporation patterns of a number of tritiated amino acids into proteins during the synchronized mammalian cell cycle.

MATERIALS AND METHODS

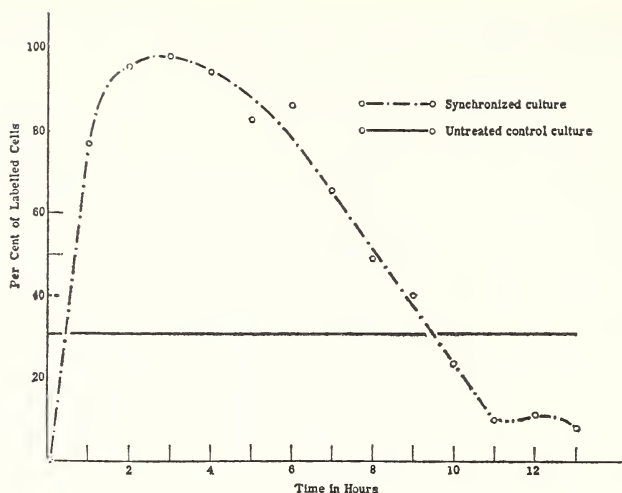
CMP human epithelial adenocarcinoma cells were synchronized in tube cultures by double blockage with excess thymidine, as described previously (6). Following removal into normal medium at the end of the second blockage, the cell population emerged as a highly synchronized unit into the S phase. H^3 -L-arginine was added for a 1-hour period at $1 \mu\text{c/ml}$ (Nuclear Chicago Batch 4, 106 mc/mM) and then fixed in Carnoy's acetic-alcohol (1:3). At the beginning of the second hour of synchrony, another tube was labeled for 1 hour. This pattern was followed for 14 hours in all, a period long enough to include S, G2, M, and the early G1 phases. Randomly growing cells and cells in the terminal stage of the second thymidine blockage were likewise exposed to labeled arginine for 1 hour.

Identical sequential labeling procedures were followed by use of H^3 -L-lysine at 20 $\mu\text{C}/\text{ml}$ and 1 $\mu\text{C}/\text{ml}$ in separate experiments (Schwarz Lot 6501, 0.8 C/mm), H^3 -4-5-leucine at 1 $\mu\text{C}/\text{ml}$ (New England Nuclear Scientific Lot 96-153A-190, 5 C/mm), H^3 -L-tryptophane at 1 $\mu\text{C}/\text{ml}$ (Nuclear Chicago Batch 2, 4.7 C/mm), and H^3 -glycine at 20 $\mu\text{C}/\text{ml}$ (Schwarz Lot 6501, 1 C/mm). Similar labeling methods were carried out with H^3 -methylthymidine and H^3 -uridine to follow incorporation patterns into nucleic acids. The detailed labeling data and results obtained with these last precursors are given elsewhere (5). Autoradiographic and staining procedures were carried out as described previously (6). Exposure times for cells labeled with tritiated amino acids were from 4 days to 2 weeks. Microscopic observations were made with bright-field and phase-contrast microscopy. Photomicrography was carried out generally with phase-contrast optics in order to reveal better cytologic detail and particularly to delimit nucleoli. Mitotic counts were made on slide preparations stained by the May-Grünwald-Giemsa technique. A minimum of 1,000 cells was counted for each slide. The percent of cells in DNA synthesis during the synchronized cell cycle and in control preparations was determined by counting at least 1,000 labeled and unlabeled nuclei in each autoradiograph labeled with tritiated thymidine for 1-hour periods. In one experiment involving H^3 -L-lysine, differential grain counts were made over cytoplasm and nucleus of 25–50 interphase cells per slide in synchronized and control preparations. Three separate cytoplasmic counts were made per unit area of cytoplasm with a square reticle in the microscope eyepiece. These 3 values were averaged to give the cytoplasmic count. The identical procedure was carried out for each nucleus by use of the same reticle. From these data the nuclear/cytoplasmic ratio of grain counts was obtained for each time period following synchronization. A similar procedure was followed for 3 control slides fixed at different times in the same experiment.

RESULTS

Synchronization

The degree of cell synchrony achieved is apparently very high, as judged by a number of criteria. There is an extremely rapid incorporation rate of thymidine into DNA in the early S phase so that 98% of cells are labeled 4 hours after initiation of the S phase. This compares with a control rate of 30%. In later stages of the cell cycle, thymidine incorporation drops to 10% (text-fig. 1). The mitotic rate in thymidine-blocked cells is 0.3% and compares with 3% mitoses in randomly growing cells and 30–70% in the first mitotic burst in synchronized cultures. Figures 1, 2, and 3 are phase-contrast photomicrographs taken from a 16 mm film sequence which demonstrates the mitotic burst in a synchronized culture. A fixed and stained preparation in figure 4 demonstrates



TEXT-FIGURE 1.—Percent of synchronized CMP tumor cells labeled with tritiated thymidine for 1 hour in normal medium after final blockage in 2.5 mM unlabeled thymidine. Time 0 hour occurs 1 hour after blocked cells are placed in normal medium. A minimum of 1,000 cells was counted for each point indicated. A cell was considered to be labeled if its nucleus had any detectable silver grains over background. The unusually high level of synchrony is especially well demonstrated in the early and mid-S phase (0–5 hours) by the rapid and almost 100% labeling rate. During the G₂ (9–11 hours) and M (11–12.5 hours) phases, synchrony drops to 85–90%.

under low power a 70% mitotic burst. Cell doubling in random populations takes about 22 hours, whereas synchronized cultures double their cell number in 2–3 hours.

Autoradiography

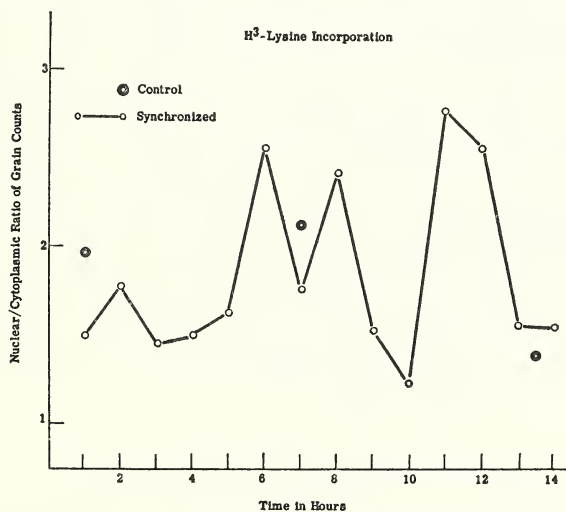
Incorporation of H^3 -L-Arginine

Random populations of CMP cells incorporate arginine into all interphase and dividing cells. There is a greater grain density over nuclei, especially nucleoli in many cells, than over the cytoplasm (fig. 5). Cells labeled in excess thymidine at the end of the second period of thymidine blockage demonstrate a relatively uniform incorporation into nuclei and cytoplasm. Some nucleoli demonstrate preferential labeling (fig. 6). In the ensuing S phase following release of the block, the pattern is essentially unaltered for the first 2 hours. This is followed by preferential incorporation into nuclei, and especially into nucleoli, during the remainder of the S phase. Some labeling is observed in the cytoplasm as well (fig. 7). During the G₂ phase just preceding the mitotic burst, there is a lack of nucleolar labeling and a return to general extranucleolar and cytoplasmic incorporation. During the M phase, dividing cells display silver grains close to the cell membranes and often associated with chromosomes. There is rarely any labeling in the spindle or interchromosomal

areas in any mitotic stages. Prophase chromosomes incorporate arginine but not preferentially, since cytoplasm is likewise labeled. There appear to be some interesting differences in patterns of arginine incorporation during the G1 phase. In a 3-hour period following the formation of daughter cells, arginine appears to be incorporated to almost the same extent into the cytoplasm as in the nucleus. In some cells, cytoplasmic labeling density is somewhat greater. Later in the G1 phase, there is an almost complete absence of cytoplasmic and chromatin labeling; silver grains are found clustered exclusively over nucleoli (fig. 8).

Incorporation of H^3 -L-Lysine

The results to be described are based on 2 separate synchrony experiments involving different isotope concentrations in the culture medium. Randomly growing CMP cells incorporate lysine into nucleoli at a somewhat greater rate than into chromatin. Radioactivity in grain density in the cytoplasm appears equivalent to that found in chromatin. By making quantitative grain counts, we demonstrated variation in absolute numbers of silver grains per unit area of nucleus (chromatin and nucleolus) and per unit area of cytoplasm between 3 control slides fixed at different time intervals. However, there was a relatively constant nuclear/cytoplasmic (N/C) ratio of approximately 2.0 (text-fig. 2).



TEXT-FIGURE 2.—The curve illustrates changes in the ratio of grain counts per unit area of nucleus/cytoplasm during the cell cycle. Nuclear grain counts also include contributions from nucleoli. The S phase extends from 0–8 hours and G2 lies between 8–10 hours. The mitotic burst occurs at 11 hours in this experiment and is followed by the G1 period. Randomly growing control cells incorporate about twice as much lysine into nuclei as in cytoplasm. Complex variations in relative incorporation rates are seen.

In early S phase cells, there is a slight preferential nucleolar and chromatin labeling compared with the cytoplasm ($N/C = 1.5-1.8$). During the middle S phase, the incorporation ratio remains essentially unaltered at 1.5. The nuclear counts include sizable contributions from the nucleolus. Grain counts in all regions decrease from 20-28 per unit area to practically zero near the late S phase ($5\frac{1}{2}$ - $7\frac{1}{2}$ hours post wash), although ratios remain high. Just prior to the G2 phase, there is a tremendous increase in labeling, especially in the nucleus. During the G2 phase, there is very little cellular incorporation of lysine. This is especially noted during the M phase in the few cells (10%) that are out of synchrony with the main population and are still in the G2 phase. Cells in mitosis generally have few grains, and when present these are over nonchromosomal areas of such cells. The only exceptions are cells in prophase, which display lysine activity associated with the chromosomes. The early G1 phase is marked by an abrupt rise in grain counts, especially in the nucleolus. Moderate activity is observed in the nucleoplasm and less in the cytoplasm.

Incorporation of H^3 -4-5-Leucine

After labeling with this protein precursor, random populations reveal a variety of autoradiographic patterns. In some cells, nuclei and cytoplasm display equal labeling. In other cells, there is predominantly nucleolar incorporation. A third type includes nuclei which are labeled so that grains are associated with both chromatin and nucleoli. Radioactive leucine is not incorporated preferentially into either the cytoplasm or nucleus during the DNA synthetic phase or the G2 phase. We observed a general distribution of silver grains throughout the entire cell. Mitotic chromosomes do not have any radioactivity associated with them when the label is added either in the G2 or mitotic phases. For the first 2 hours after the mitotic burst in very early G1 phase, there is again the random labeling pattern mentioned above. However, an abrupt change first occurs 3 hours after the onset of the G1 phase. At this time, we detected selective uptake into chromatin and nucleoli and barely any cytoplasmic incorporation. This burst in incorporation into nuclear proteins continues for 3 hours in the G1 phase and then shifts exclusively to nucleolar labeling. At this time, this particular experiment was terminated so that we did not follow the pattern further.

Incorporation of H^3 -L-Tryptophane

The addition of tritiated tryptophane to control cells does not produce any unusual incorporation patterns in the S, G2, and M phases. There is a somewhat random incorporation into the cytoplasm and nucleus of interphase cells in addition to some nucleolar labeling (fig. 9). Cells fixed in the process of mitosis also contain label, mostly around the cell margins and to a lesser extent over chromosomes. There are never any silver

grains in the region of the mitotic spindle. Synchronized cells which are labeled in the premitotic stages (S and G₂) reveal a heavy cytoplasmic uptake in addition to simultaneous incorporation into nuclei (fig. 10). In the subsequent G₁ phase, cytoplasmic incorporation ceases and nucleoli continue to incorporate tryptophane into protein, although at a much lower rate than in the preceding premitotic phases. There appear to be variations in this pattern during the G₁ phase similar to those mentioned in the case of leucine, but we have not studied the preparations in sufficient detail to make any further statements at this time.

Incorporation of H³-Glycine

Glycine is incorporated into cytoplasmic and nuclear proteins of control, blocked, and synchronized cells at all stages of the cell cycle. Cells in mitosis also display radioactivity but not over the chromosomes. The only period during the cell cycle when any preferential labeling occurs is in the G₁ phase. During this portion of the cycle, there is a prominent deposition of silver grains over many nucleoli. Nucleolar incorporation of glycine is superimposed on some extranucleolar and cytoplasmic labeling.

DISCUSSION

The employment of highly synchronized cell cultures has provided us with a valuable tool by which to analyze the complex events attending the biosynthesis of nucleoproteins during the mammalian cell cycle. Because of the limitations of space, it is impossible to discuss the RNA and DNA synthetic patterns observed in the synchronized system employed in the present study. Since a fuller report is given elsewhere (5), the reader is referred to the cited reference and to the introductory remarks made at the beginning of the present report.

The results described here indicate that a variety of amino acids are incorporated into dividing mammalian cells in complex patterns during selected phases of the cell cycle. Arginine was found to be incorporated into chromatin and nucleoli during most of the S phase. Since histones are known to be complexed with chromosomal DNA and both of these macromolecules are synthesized during the S phase (7, 8), it is likely that the proteins labeled with arginine are histones. Some of the extranucleolar protein containing labeled arginine may not be associated with DNA, since there is still arginine incorporation into the nucleoplasm in the G₂ phase. Prensky and Smith (9) also reported turnover of a major arginine-rich nuclear protein during one cell division cycle. The fact that nucleolar incorporation of arginine ceases during the G₂ phase would further support the idea that the nucleolar-associated histone is bound to intranucleolar DNA. The presence of DNA in this organelle is revealed in a number of studies based on biochemical analyses, cytochemical

staining, and EM studies (*cf* 5). According to recent autoradiographic studies, there are likely 2 metabolically distinct DNA fractions in the nucleolus (active or euchromatin and inactive or heterochromatin), the first of which functions as a template for RNA synthesis (5).

The G1 phase appears to be another period marked by nuclear and nucleolar protein synthesis, although amino acid turnover may account for part of this incorporation. Not only is arginine incorporated into nucleoli of G1 cells, but lysine, leucine, tryptophane, and glycine are all preferentially or selectively incorporated into this organelle during the G1 phase. We cannot state on the basis of this study how many different nucleolar proteins may be incorporating these amino acids. According to recent reports, there are a number of heterogeneous arginine-rich histones which are likewise rich in lysine, glycine, and leucine (10). In view of the concordance of the amino acid composition data with our results on amino acid incorporation patterns, probably the nucleolus is a repository for arginine-rich histones which are synthesized in the G1 phase in multiplying cell populations. In further support of this hypothesis is the report that histones control the "readout" of nucleolar-associated DNA (11). It is suggested that appropriate biochemical analyses should be carried out on isolated nucleoli of synchronized G1 cells to categorize these histones and help understand their biological roles.

There is a complex pattern of lysine incorporation during the cell cycle. One point we should emphasize is that 1.5 to 3.0 times more lysine is incorporated into nuclei than into cytoplasm of synchronously dividing cultures. There appears to be a sudden burst in uptake of lysine into nuclei just prior to the G2 phase. The significance of this finding needs to be explored further, especially relative to the problem of histone complexing with DNA. It is also conceivable that this lysine-rich protein may be associated with the events necessary for triggering mitosis. Nuclear-incorporated lysine is partitioned between the nucleoplasm (chromatin) and the nucleolus, which supports the idea that at least 2 different lysine-rich proteins are present.

RESUMEN

Células tumorales humanas epiteliales CMP en cultivo fueron sincronizadas en el ciclo celular empleando un doble bloqueo secuencial con timidina en exceso. Se logró un elevado nivel de sincronismo de acuerdo a varios criterios y se produjeron porcentajes de desencadenamientos mitóticos tan elevados como del 70 por ciento. Se emplearon técnicas autorradiográficas en conjunción con arginina, lisina, leucina, triptofano y glicina tritadas para detectar los patrones de incorporación intracelular. La arginina se incorpora en la cromatina y los nucleolos durante la fase S y en la cromatina durante la fase G2. La fase G1 se caracteriza por una incorporación preferencial o selectiva de todos los 5 aminoácidos en los nucleolos. Por lo general, esto ocurre unas pocas horas después de completarse la mitosis. Se sugiere que este desencadenamiento G1 de incorporación se debe, particularmente, a la síntesis en el nucleolo de histonas ricas en arginina. Hay 1,5-3,0 veces más lisina incorporada

en los núcleos (cromatina y nucleolo) que en el citoplasma durante el ciclo celular. El período S terminal se caracteriza por un brusco aumento de la lisina asociada con la cromatina y el nucleolo. Estas proteínas ricas en lisina pueden ser de significación en relación con la formación del complejo de la histona con el ADN o en el desencadenamiento de la mitosis.

REFERENCES

- (1) TAYLOR, J. H.: Nucleic acid synthesis in relation to the cell division cycle. *Ann NY Acad Sci* 90: 409-421, 1960.
- (2) EVANS, H. J.: Uptake of ^3H -thymidine and patterns of DNA replication in nuclei and chromosomes of *Vicia faba*. *Exp Cell Res* 35: 381-393, 1964.
- (3) SISKEN, J. E., and MORASCA, L.: Intrapopulation kinetics of the mitotic cycle. *J Cell Biol* 25: 179-189, 1965.
- (4) HUANG, P. C., and BONNER, J.: Histone, a suppressor of chromosomal RNA synthesis. *Proc Nat Acad Sci USA* 48: 1216-1222, 1962.
- (5) KASTEN, F. H., and STRASSER, F. F.: Nucleic acid synthetic patterns in synchronized mammalian cells. *Nature (London)* 211: 135-140, 1966.
- (6) KASTEN, F. H., STRASSER, F. F., and TURNER, M.: Nucleolar and cytoplasmic RNA inhibition by excess thymidine. *Nature (London)* 207: 161-164, 1965.
- (7) ALFERT, M., and GESCHWIND, I.: A selective staining method for the basic proteins of cell nuclei. *Proc Nat Acad Sci USA* 39: 991-999, 1953.
- (8) BLOCH, D. P., and GODMAN, G. C.: A microphotometric study of the syntheses of deoxyribonucleic acid and nuclear histone. *J Biophys Biochem Cytol* 1: 17-28, 1955.
- (9) PRENSKY, W., and SMITH, H. H.: Incorporation of ^3H -arginine in chromosomes of *Vicia faba*. *Exp Cell Res* 34: 525-532, 1964.
- (10) HNILICA, L. S., and BESS, L. G.: The heterogeneity of arginine-rich histones. *Anal Biochem* 12: 421-436, 1965.
- (11) LIAU, M. C., HNILICA, L. S., and HURLBERT, R. B.: Regulation of RNA synthesis in isolated nucleoli by histones and nucleolar proteins. *Proc Nat Acad Sci USA* 53: 626-633, 1965.

PLATE 46

Figures 1-3 are selected stills from a 16 mm film taken by phase-contrast, time-lapse cinematography. The film was made from a CMP cell culture grown in the Rose chamber and synchronized by the excess thymidine method.

FIGURE 1.—Appearance of cells in the S phase, $7\frac{1}{2}$ hours after transfer to normal medium.

FIGURE 2.—Same field $3\frac{1}{2}$ hours later in the G2 period (11 hours). *Note* the rounding up of some cells just undergoing mitosis.

FIGURE 3.—Same field 2 hours later during the M phase (13 hours). *Note* the mitotic burst demonstrated by the numerous rounded cells.

FIGURE 4.—The mitotic burst from another synchrony experiment is revealed here in a fixed preparation stained by the May-Grünwald-Giemsa method. Several hundred cells are in this field, which has a mitotic count of 70%.

Photomicrograph from an experiment by Miss Susan House.

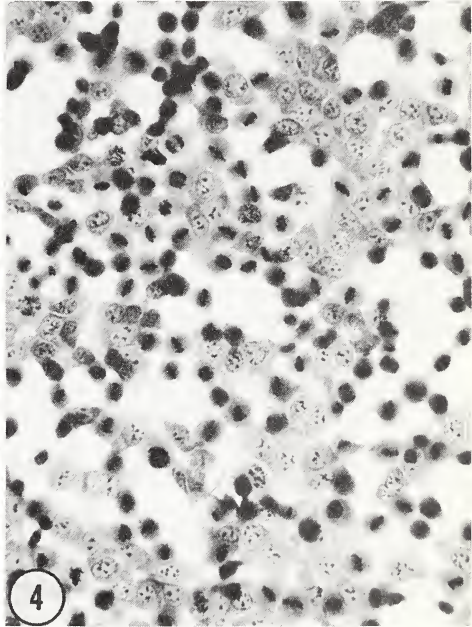
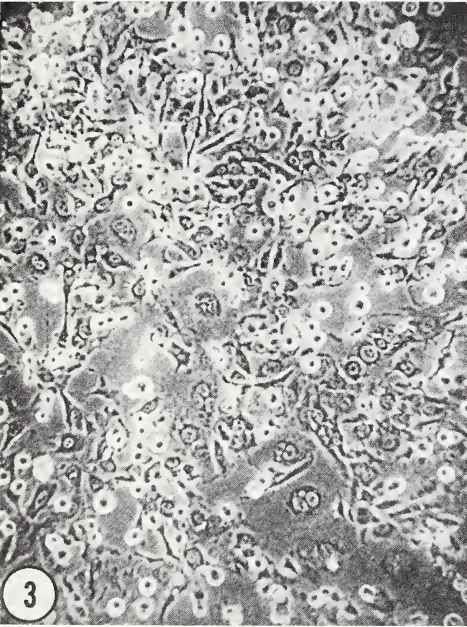
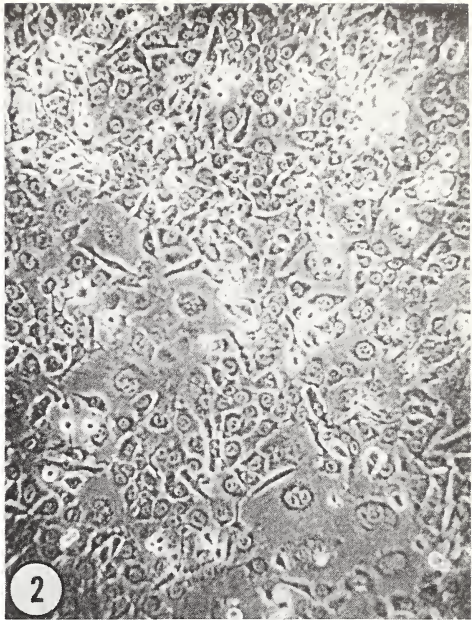


PLATE 47

Phase-contrast photomicrographs taken on 4×5 Tri-X film with the aid of a #53 orange filter to enhance contrast. All tritiated amino acids are added to cultures for 1 hour prior to fixation. Autoradiographs prepared by use of the AR10 stripping film method followed by staining with hematoxylin and eosin.

FIGURE 5.—Randomly growing cells labeled with H^3 -L-arginine. Incorporation of isotope appears in cytoplasm, chromatin, and nucleoli. $\times 800$

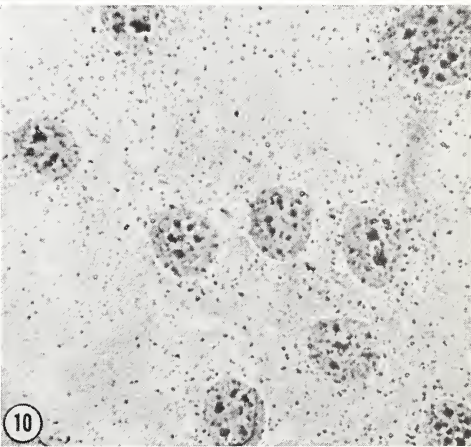
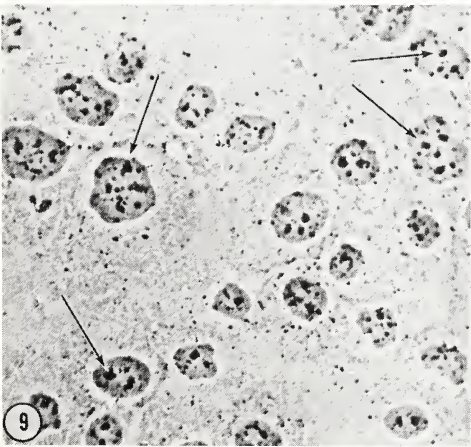
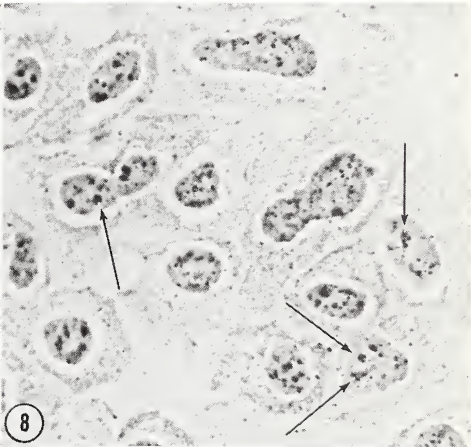
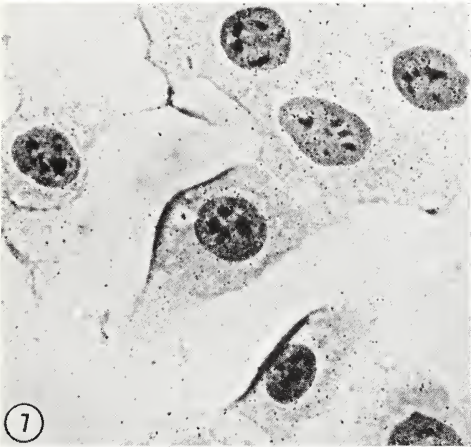
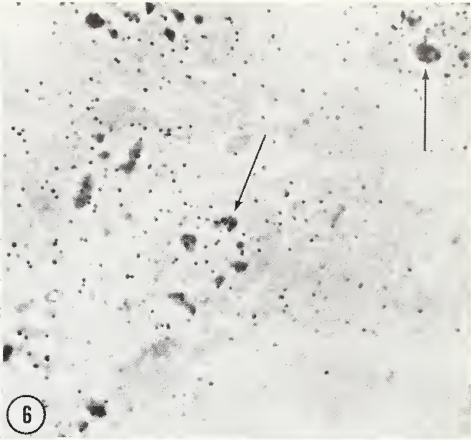
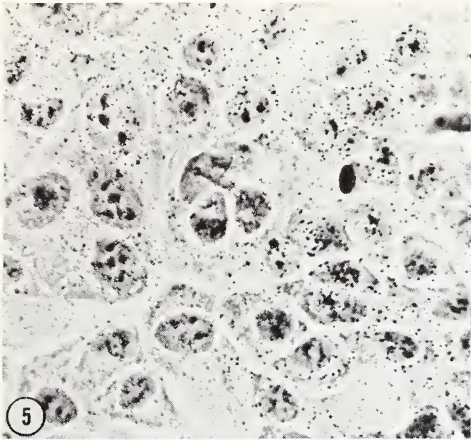
FIGURE 6.—Cells labeled with H^3 -L-arginine while in the second thymidine blockage. Protein synthesis is apparently unaffected by the blockage of DNA synthesis. Grains appear in cytoplasm and nucleus. *Arrows* indicate labeled nucleoli. $\times 1,260$

FIGURE 7.—Incorporation of tritiated arginine into synchronized cells during the S phase. Although the cytoplasm is definitely labeled, there appears to be more nuclear label in a few cells. $\times 800$

FIGURE 8.—Photomicrograph demonstrates selective uptake of arginine into nucleoli of G1 phase cells 14 hours after the onset of synchrony. *Arrows* point to nucleoli which contain well-delimited associations of silver grains. $\times 800$

FIGURE 9.—Randomly growing cells labeled with H^3 -L-tryptophane. Some activity appears in the cytoplasm but the principal labeling is in nuclei, especially nucleoli (*arrows*). Such results support the idea that a tryptophane-rich histone is localized in the nucleolus. $\times 800$

FIGURE 10.—This field is from a middle S phase preparation labeled with H^3 -L-tryptophane. Uptake into protein apparently occurs in all cellular regions during this period of the cell cycle. Selective incorporation into nucleoli occurs in the G1 phase. $\times 800$



DISCUSSION

Feinendegen: May I add a few points pertinent to this session? After administration of a labeled RNA precursor, the autoradiographic labeling pattern of nucleoli in mammalian cells shows a rather wide range of variation, such as Dr. Kasten just demonstrated to occur during the first part of the "S phase" of the cell cycle. For my own attempts at analyzing the significance of these variations a particularly suitable cell strain was selected, and was kindly supplied by Dr. Barski's laboratory in Villejuif (France). This "hybrid type" clone M #6, obtained from two different mouse cell lines, has unusually large nucleoli with diameters up to $10\ \mu$, and many cells of this strain have only one large nucleolus. The cells were grown in a non-synchronized culture in monolayer and were labeled with $\text{H}^3(5,6)$ -uridine or $\text{H}^3(5)$ -cytidine for 1, 5, 10, or 20 minutes. In some experiments, a chase with $20\ \mu\text{g}$ of non-labeled nucleoside per ml followed up to 12 hours. An examination of the labeling pattern by autoradiography (AR-10, Kodak, stripping film) showed the following:

1. The grain density above the nucleoli (whether single or double per cell) rose linearly with the total nuclear grain density. However, at any level of total nuclear grain density, except at the highest, the ratio of nucleolar grain density to grain density of extranucleolar chromatin varied from below 1 to 10, and did not appear to be random, since sister cells, which are probably equal genetically, showed, within the range of distribution, nearly the same ratio. This suggests that the nucleolar function with regard to precursor incorporation into RNA relative to the corresponding function of the extranucleolar chromatin is determined genetically, and varies even in cells derived from one strain. It is known that the total rate of RNA precursor incorporation rises with progression of the cell cycle toward mitosis. It appears therefore that the variation of the relative labeling intensity of the nucleolus is largely independent of the cell cycle.

2. The relative labeling intensity of the nucleolar center only, as a fraction of the total nucleolar grain density, also varied considerably and independently of the total nuclear grain density except at the upper third level. And also here, pairs of sister cells showed similar labeling patterns. This clearly indicates that RNA precursor incorporation in mammalian cells also is not a random process, whatever RNA fraction is involved, and that DNA segments engaged in RNA synthesis at any given time are genetically specified and differ with regard to the nucleolus even in cells obtained from one strain. I should add that at no time were there single nucleoli completely devoid of label. Since the rate of progression of sister cells through the generative cycle is presumably not strictly equal, one is led to suggest that the factors determining the particular sites of RNA synthesis do not alter rapidly and probably persist through fairly long sequences of the generative cycle.

3. The relative labeling intensity of 2 nucleoli in one cell, or the difference in grain density between them, also showed a wide range of variation, independent of total nuclear grain density or of nucleolar area or of absolute grain counts over a wide range. Again this variation occurred, regardless of whether the cells were labeled for 1, 5, 10, or 20 minutes. However, in this instance, sister cells were not similar. This suggests that, within the total chromatin, DNA segments involved in RNA synthesis at a given time are distributed between two nucleoli unequally and probably at random.

4. After labeling with H^3 -uridine for 20 minutes and a chase with cold uridine ($20\ \mu\text{g}$ per ml medium) to 3 hours, the degree of nucleolar grain density again rose with total cell labeling and existing variations were shared by sister cells. Furthermore, the ratio of grain density of the nucleolus to extranucleolar chromatin was signif-

icantly altered in favor of the nucleoli. Simultaneously, the relative number of nucleoli with an elevated grain density over the nucleolar center increased, and the difference in grain density between two nucleoli in one cell diminished. This obviously confirms that labeled RNA is present in the nucleolus for a longer period than in the extra-nucleolar chromatin area. This could result from an RNA fraction with a slow turnover. In addition, the data suggest that continuous incorporation of precursor, or rearrangement of labeled RNA or its fragments into nucleoli, occurred at a time when chromatin labeling diminished.

Taking the nucleolus and its peripheral chromatin of this heteroploid cell as a definite reference point within the total nuclear chromatin in interphase, I reiterate that these observations emphasize that RNA precursor incorporation at a given time is controlled by genetic factors which obviously vary in different cells of this particular strain. Apparently, RNA synthesis is confined mainly to selected chromatin areas per cell at a given time, and template function does not shift from one chromatin area in the nucleus to another within less than 20 minutes and probably not over a period considerably longer. It perhaps does not occur even within the period of one cell cycle.

Kasten: I think it is profitable to have large nucleoli to work with, but ultimately I think we will have to go to electron microscopic autoradiography to study in more detail the problem of intranucleolar incorporation patterns. This is the more logical level to work at, but I certainly think your material appears very useful for light microscope studies.

Feinendegen: I think you are right there. But electron microscopic autoradiography is far from being a quantitative technique, and we tried to express our data quantitatively, that is, in number of grains per unit area in sister cells.

Kasten: I might add that we have EM-autoradiographic preparations of synchronized cells fixed at different stages of the cycle, but do not have any results to discuss at this time.

Bianchi: Dr. Kasten, when you used thymidine to label the cells, you obtained a very interesting pattern consisting of labeling all over the nucleus except for the areas around the nucleolus; at the end of this phase do you find the reverse pattern? That is, do you find the rest of the nucleus free of label but the areas around the nucleolus labeled?

Kasten: No, we have not seen this pattern. During the DNA synthetic phase we find two times when the nucleolus synthesizes DNA preferentially. The first time is early in the cycle when a perinucleolar space free of grains is observed around each nucleolus, and the second time is about an hour later in mid-S phase when there is no unlabeled space around the labeled nucleolus.

Lettré: I think that Dr. Kasten misunderstands me when he says that I think the chromatin has to always be present in the form of extended filaments. Was this a Feulgen reaction which you demonstrated?

Kasten: Yes, it was a modified Feulgen reaction, a fluorescent-Feulgen reaction.

Lettré: The structures looked so fuzzy and a little bit indistinct.

Kasten: Well, this is the way the DNA looks in these cells.

Lettré: In our preparations the chromatin looks more distinct and we can see filaments. We have the fuzzy aspect only after swelling of the cells.

Love: While I agree with Dr. Kasten that the earliest changes in virus-infected cells are frequently seen in the nucleoli, I would like to stress two points. One is that this occurs with RNA viruses as well as with DNA viruses. For example, infection with parainfluenza virus produces changes in the nucleolus (Love and Suskind, *Exp Cell Res* 24: 521-526, 1961). Secondly, some DNA viruses, as Dr. Bernhard has shown with SV40, do replicate early in the nucleolus and produce early changes in the nucleolar RNA of the nucleolini but not in the nucleolar DNA (Love and Wildy, *J Cell Biol* 17: 237-254, 1963).

Kasten: I am familiar with Dr. Bernhard's work, and I am sure he excuses me for not mentioning his work in the brief time available for my presentation. Did you

mean to suggest that with RNA viruses you get DNA inclusions within the nucleolus, or did you simply mean morphological aberrations?

Love: No, we get changes in the so-called nucleolini. Disappearance of the nucleolini is one of the earliest stages. And that is RNA, not DNA.

Swift: You have presented some interesting data on autoradiographs made at different stages in the mitotic cycle, and you have mentioned the variables of sensitivity and conditions of development. But you have also made some remarks about the incorporation rates of the nucleolus and other cell structures. I would like to ask if these are merely based on visual estimates, or whether you have made corrections for the concentration of material in the cell beneath the emulsion, for the section thickness, and also for the tissue density (because of self absorption). I don't see how you can conclude that the nucleolus shows preferential incorporation of amino acids into proteins until you can weigh these variables. The nucleolus frequently contains the most concentrated protein found anywhere in the cell, and such factors need to be considered before you can estimate relative specific activity.

Kasten: Does your question relate to the amino acid or nucleoside incorporation experiments?

Swift: Both.

Kasten: Let me just ask one more question before I try to answer you. Did you mean to suggest that the factors of thickness and density present worse problems in synchronized culture experiments than in routine autoradiography experiments published by many workers?

Swift: I think what you are trying to do deserves a careful autoradiographic study, and visual estimates alone may be misleading.

Kasten: I agree in principle, but in this case there were no real absolute quantitative data meant to be presented; these were patterns of incorporation. As such, there may be some variation. What I have emphasized are simply the most prominent changes in the cell cycle. These changes are of a magnitude which I think the human eye and an instrument might not differ by too much. The preferential incorporation of amino acids into nucleoli during G1 is an example of one of these striking changes.

Altered Patterns of RNA Metabolism in Liver Cells Following Partial Hepatectomy and Thioacetamide Treatment¹

RUTH G. KLEINFELD, *Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, New York 13210*

SUMMARY

The RNA metabolism of regenerating rat liver parenchymal cells at different periods following partial hepatectomy was contrasted with that of liver cells responding to thioacetamide. The patterns of incorporation of H³-cytidine into nucleolar and cytoplasmic RNA were quantitatively analyzed by autoradiographic techniques. Distinct shifts in the patterns of incorporation and turnover into nucleolar RNA were seen at 6, 12, and 20 hours, following partial hepatectomy. Total incorporation into the nucleolus of the thioacetamide-affected cell showed a 25-fold increase over control values, whereas cytoplasmic labeling remained comparable to that

at the control level. Animals taken off the drug for 4 days continued to show an elevated rate of incorporation into nucleolar RNA, and cytoplasmic labeling was increased. Thioacetamide-treated rats subjected to partial hepatectomy showed a shift in nucleolar and cytoplasmic labeling patterns to patterns resembling those of regenerating liver cells of untreated animals. The possibility that thioacetamide activates the rate of precursor ribosomal RNA synthesis without affecting other processes involved in transfer to the cytoplasm and ribosome formation was considered.—*Nat Cancer Inst Monogr* 23: 369–378, 1966.

AS INFORMATION accumulates concerning the roles of the different ribonucleic acids (RNA) in protein synthesis, information relating to the metabolism and morphology of the various RNA-containing components of the cell during periods of transition seems particularly relevant to our understanding of nuclear-cytoplasmic interactions. In the present report the RNA metabolism of regenerating rat liver during different times of the transition period (the period during which cells are preparing for division) is compared with that of liver cells responding to thioacetamide (TA), a drug that induces a rapid and specific effect on nuclear RNA metabolism (1–6), particularly affecting the nucleolus.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

METHODS

Partial hepatectomy.—Partial hepatectomy was performed according to the procedure of Higgins and Anderson (7), whereby two thirds of the liver is removed. Each animal received a single intraperitoneal injection of H^3 -cytidine at a dose of $150\ \mu\text{c}/100\ \text{g}$ body weight, 6, 12, or 20 hours after surgery. Animals from each group were sacrificed at intervals $\frac{1}{2}$ hour to 12 hours following isotope administration. Laparatomized animals served as controls.

Thioacetamide.—Animals were fed a diet of ground Purina chow containing 0.04% TA for 14 days. A single intraperitoneal injection of H^3 -cytidine ($150\ \mu\text{c}/100\ \text{g}$ body weight) was given as indicated. Actinomycin D was given intraperitoneally at a dose of $6\ \mu\text{g}/100\ \text{g}$ body weight. Control animals were fed ground Purina chow minus TA. Food amounts were restricted to match the mean daily intake of the TA-fed groups. Liver samples were fixed in 1:3 acetic acid-ethanol for 4 hours.

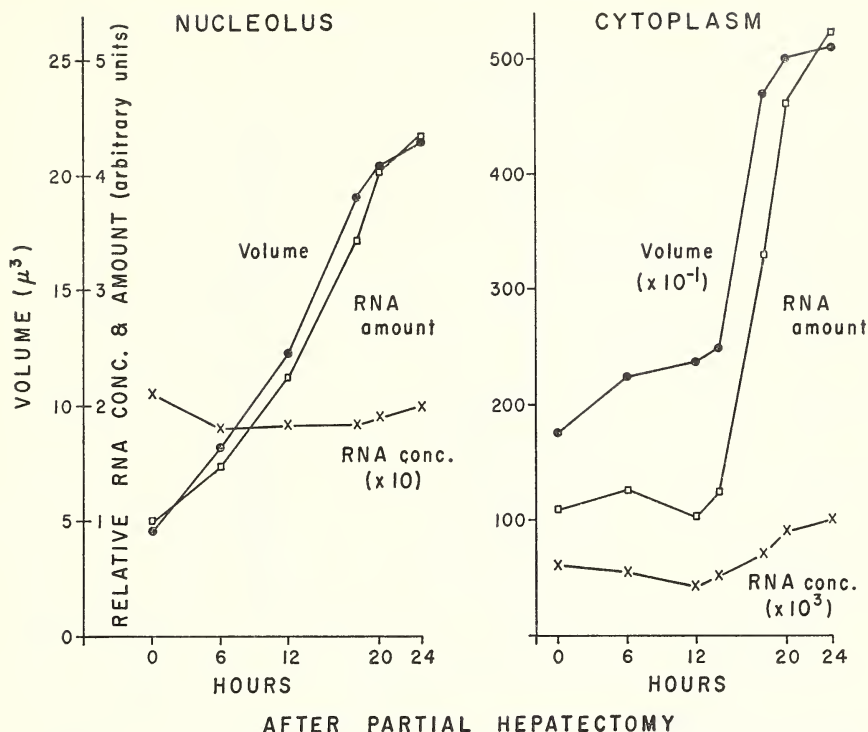
Autoradiography.—Sections were extracted and prepared for autoradiography as previously described (4). Incorporation of H^3 -cytidine into RNA was quantitated by counting grains over the nucleolus, non-nucleolar portions of the nucleus (chromatin), and cytoplasm in individual cells of deoxyribonuclease-treated sections. A camera lucida drawing then was made on standardized paper. The relative area of each component was obtained by weighing the cutouts and the data were expressed as grains per unit area, or total grains per component (grains/unit area \times volume).

Photometric measurements.—The relative concentrations of nucleolar and cytoplasmic RNA were estimated by cytophotometric measurements of azure B dye binding at pH 4.0 on deoxyribonuclease-treated sections, as previously described (3, 4). Concentration was calculated by dividing the mean extinction at $590\ \text{m}\mu$ by section thickness. Total dye amounts were calculated by multiplying concentration by the computed volume. Cytoplasmic volumes were determined by the Chalkley method (8).

RESULTS

The heterogeneity among parenchymal cells in different zones of the liver lobule should be taken into account in any cytological study. Following partial hepatectomy the cells of the peripheral and midzone regions respond several hours before the cells of the central vein area (9-11). In contrast, TA primarily affects cells of the central vein areas (3, 4). In this study, measurements of regenerating liver cells were selectively restricted to the periportal areas and in the TA-treated animals to cells around the central vein areas.

The changes in volume and RNA amounts of nucleoli and cytoplasm during the first 24 hours following partial hepatectomy are graphed in

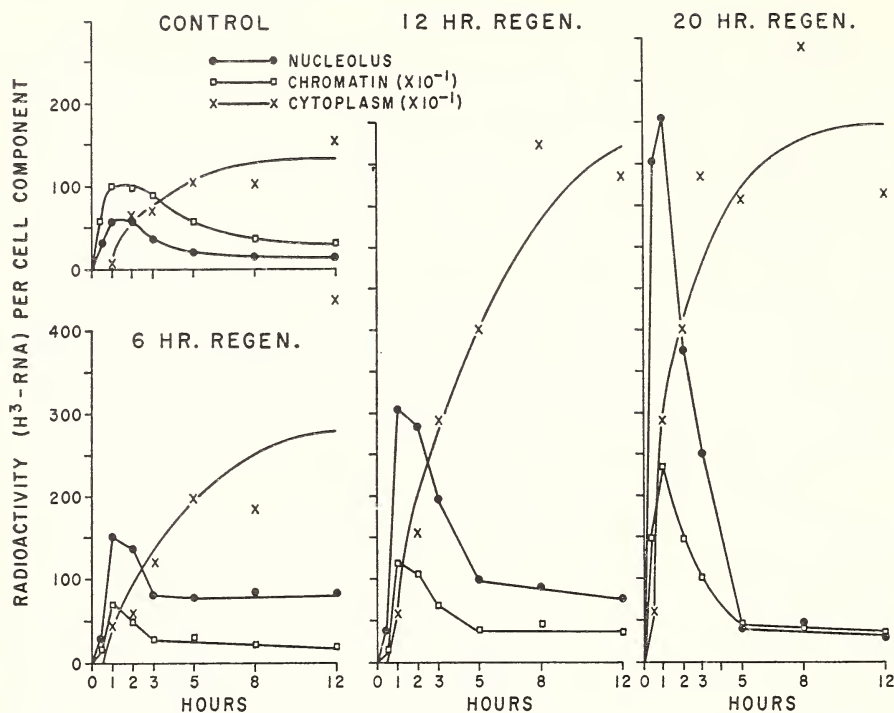


TEXT-FIGURE 1.—Volumes and relative amounts of RNA (azure B) of the nucleolus and cytoplasm in rat liver cells after partial hepatectomy.

text-figure 1. There is a rapid rise in nucleolar size and RNA content reaching approximately 4 times the control value at 20 hours. Cytoplasmic volumes increase sharply 15 to 20 hours postoperatively. This is accompanied by a fourfold increase in RNA amounts.

Autoradiographic studies of RNA labeling patterns of the nucleolus and chromatin of several cell types have been interpreted as showing at least two fractions, a rapid turnover fraction and a slow turnover fraction (4, 10, 12). The presence of a third fraction with a distinctive rate of turnover has recently been suggested (13).

The time course of incorporation of H^3 -cytidine into nucleolar, chromosomal, and cytoplasmic RNA (plotted as total radioactivity/component) after partial hepatectomy is shown in text-figure 2. In the control rat liver, the ratio of incorporation into the rapid turnover fraction (peak value at 1–2 hours) to the slow turnover fraction (leveling off value at 8–12 hours) is around 3:1. Incorporation into cytoplasmic RNA reaches a plateau level at 3 to 5 hours. Following partial hepatectomy, peak incorporation into nucleolar RNA is markedly increased at 12 and 20 hours. Twenty hours postoperatively peak incorporation of radioactive precursor into nucleolar RNA is reached earlier, and the ratio of the peak

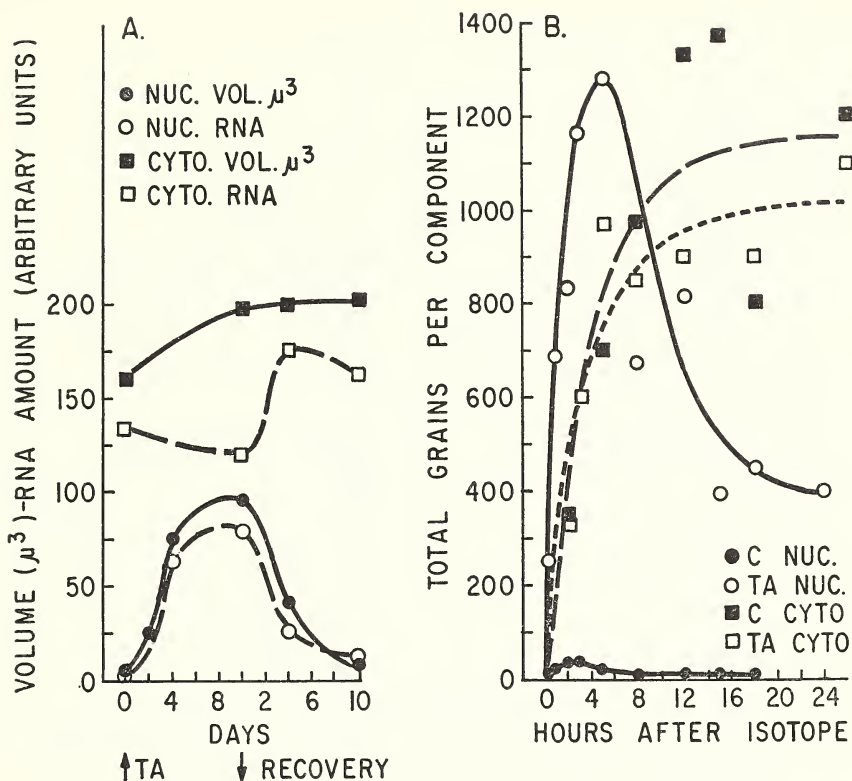


TEXT-FIGURE 2.—Changes in total grain count (grain density/unit area \times volume) in the nucleolus, chromatin, and cytoplasm at time intervals following a single injection of H³-cytidine. Control liver is compared with liver samples taken from animals injected with isotope 6, 12, or 20 hours after partial hepatectomy.

value to the leveling off value is 20:1 which markedly deviates from the control pattern of 3:1.

The effect of TA on nucleolar and cytoplasmic volumes, RNA amounts, and precursor incorporation is shown in text-figure 3. The drug induces a rapid rise in nucleolar size and RNA content, while cytoplasmic RNA amounts decrease slightly. When TA treatment is discontinued, cytoplasmic RNA levels increase and nucleolar RNA decreases. Incorporation of H³-cytidine into the nucleolus is greatly increased in the drug-treated liver cell, while cytoplasmic labeling remains comparable to that of the control (text-fig. 3B). The ratio of the peak nucleolar radioactivity (at 5 hours) to the leveling off value (15–18 hours) is approximately 3:1. Thus, not only are increased amounts of nucleolar RNA being synthesized, but obviously increased amounts are being lost from the nucleolus.

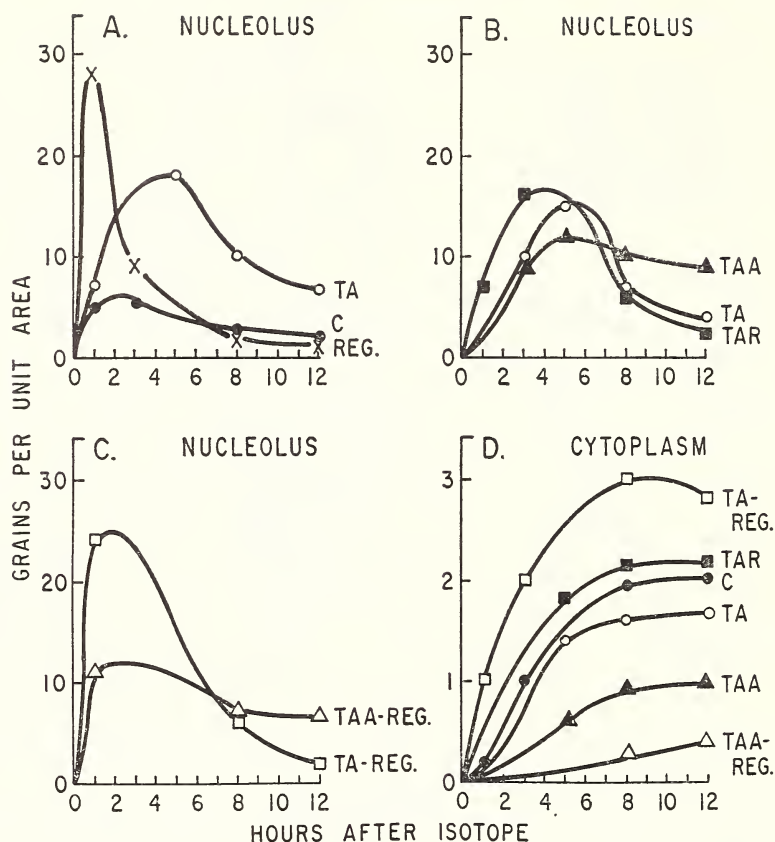
It seemed of interest to compare nucleolar and cytoplasmic labeling in liver cells of TA-treated rats when cytoplasmic RNA amounts are increasing, *i.e.*, during the early recovery period after drug withdrawal and



TEXT-FIGURE 3.—(A) Changes in volumes and RNA amounts (azure B) of the nucleolus and cytoplasm of liver cells during 10 days of TA treatment and during a 10-day recovery period. (B) Changes in total grain count (grain density/unit area \times volume) in the nucleolus and cytoplasm of liver cells at intervals after a single injection of H^3 -cytidine given to rats fed TA for 14 days. Data taken from Koulisch and Kleinfeld (4).

after partial hepatectomy (14). Results of a preliminary study are shown in text-figure 4.

After 4 days of recovery, incorporation into nucleolar RNA (grain density/unit area) is still much higher than that of control rates (text-fig. 4B). Cytoplasmic labeling is increased above that of both TA and control levels (text-fig. 4D). Cells of TA-treated animals subjected to partial hepatectomy show a 50% increase in nucleolar grain density above the TA-treated cells (text-fig. 4C). The peak level is reached earlier and the ratio of the peak value to the leveling off value resembles that of the regenerating liver cell pattern (*cf* TA-Reg. in 4C to Reg. in 4A). Cytoplasmic labeling starts earlier and is also increased (text-fig. 4D). Actinomycin blocks the characteristic loss of nucleolar label and depresses incorporation into cytoplasmic RNA in both the TA recovery and TA-regenerating liver cells (ext-fig. 4B, C, and D).



TEXT-FIGURE 4.—(A) Comparison of the time course of incorporation of H^3 -cytidine (expressed as grain density/unit area) into nucleolar RNA of liver cells from control (C), 20-hour regenerating (Reg) and 14-day TA-treated (TA) animals after a single injection of H^3 -cytidine. (B) Changes in nucleolar labeling with time after isotope administration in animals treated with TA for 14 days (TA), in animals 4 days after drug withdrawal (TAR), and in animals given actinomycin (2 hours after isotope administration) after a 4-day recovery period (TAA). (C) Changes in nucleolar labeling with time after isotope injection in TA-treated animals 24 hours after partial hepatectomy (TA-Reg), and in TA-treated animals 24 hours after partial hepatectomy who received actinomycin at 8-hour intervals postoperatively (TAA-Reg). Isotope was given 6 hours after the third injection of actinomycin. The level of nucleolar labeling indicates that recovery from actinomycin inhibition had occurred. (D) Changes with time of cytoplasmic labeling in the experimental animal groups as described in 4B and 4C.

DISCUSSION

In this study, RNA synthesis in individual liver cells of animals subjected to partial hepatectomy and treatment with thioacetamide has been examined with the objective of evaluation of nucleolar and cytoplasmic labeling patterns during shifts in cellular function.

Regenerating Liver

The period following partial hepatectomy and preceding the first burst of mitosis may be roughly divided into three phases [for review *see* (15)]. The initial 6-hour period probably involves "selective" derepression and repression of gene transcription (16) which then initiates the following sequence of metabolic phases. The period 6–12 hours may be characterized as one of reorganization as some of the synthetic pathways operative in the nondividing cell breakdown and new biosyntheses are switched on. The period 12–24 hours is one of peak synthetic activity. The first burst of mitosis occurs at 24–32 hours involving 20–40% of the parenchymal cells.

The autoradiographic results may be summarized as follows: At 6 hours postoperatively, there is a twofold increase in nucleolar and cytoplasmic labeling. The presumed changes that occur at the genetic level as new messenger RNAs are transcribed are not reflected in altered RNA labeling of the non-nucleolar portions of the nucleus. At 12 hours the cell is entering a period of intense biosynthetic activity linked to cell replication. Incorporation into nucleolar and cytoplasmic RNA at this time shows a sixfold increase. At 20 hours incorporation into nucleolar RNA increases further, while cytoplasmic labeling remains comparable to that in the 12-hour group. Since nucleolar size and RNA content are approaching a maximum, one might expect the rate of synthesis and rate of loss of nucleolar material to be in some sort of equilibrium at this time. As shown in text-figure 2, the rate of loss of nucleolar label is greatest in the 20-hour group, whereas total cytoplasmic labeling remains comparable to that of the 12-hour group level.

Thioacetamide

It has been shown that the increased size of the nucleolus induced by thioacetamide is accompanied by increased rates of synthesis of RNA (4–6) corresponding to the high molecular weight ribosomal precursor RNA (6, 17). Radioautographic studies [text-fig. 3*B* and (4)] show that incorporation into nucleolar RNA is increased 25 times over control values. There is a proportionate loss of radioactivity with time, whereas cytoplasmic labeling remains comparable to control levels. When the drug is withheld from the animals for 4 days, incorporation rates remain high and cytoplasmic labeling increases. When TA-treated animals are subjected to partial hepatectomy, the patterns of nucleolar and cytoplasmic labeling resemble those of the regenerating liver cells of untreated animals (text-figs. 4*C* and *D*).

There is now substantial evidence to support the hypothesis that the nucleolus is the site of synthesis of ribosomal RNA (18–20). Whether nucleolar activity is exclusively linked with ribosome biosynthesis has not been unequivocally resolved.

It has generally been shown that nucleolar and cytoplasmic RNA labeling patterns are related, that loss of nucleolar label is followed by increased cytoplasmic labeling, and that newly formed nucleolar RNA consists primarily of ribosomal precursor RNA (18, 19). If "pre-ribosomal" RNA directs and controls the rate of ribosomal protein synthesis leading to the formation of mature ribosomes (21), then one might expect a fairly consistent ratio to exist between loss of nucleolar labeling and cytoplasmic labeling. If, however, the regulation of ribosomal protein synthesis and the transfer of ribosomes to the cytoplasm are relatively independent of "pre-ribosomal" RNA synthesis, then the kinetics of nucleolar and cytoplasmic labeling could be quite complex.

A comparison of the 12- and 20-hour regenerating liver series indicates that the relationship between loss of nucleolar label and total cytoplasmic labeling is not a simple one. In the TA-treated liver cell, where it has been shown that newly labeled RNA is primarily ribosomal precursor RNA (6, 17), the large loss of nucleolar label does not reach the cytoplasm (text-fig. 3B). It has recently been reported that thioacetamide markedly alters nuclear enzyme activities, *i.e.*, increase in RNA polymerase activity (22), a marked increase in latent ribonuclease activity (23), and the presence of a suppressor of a ribonuclease which cleaves ribosomal precursor RNA (6). Since the effect of TA is readily reversible, and partial hepatectomy may in part counteract the TA effect, it would seem that the basic steps in the transfer of RNA to the cytoplasm and ribosome formation are not blocked.

Let us examine the idea that TA does not alter normal biosynthetic processes *per se*, but rather activates the initial phase of ribosomal precursor RNA transcription, *i.e.*, TA increases the number of sites open for transcription or the rate of transcription. If ribosomal RNA synthesis is independent of ribosomal maturation and release, and the latter processes proceed at approximately control rates during treatment with TA, this would explain (a) the pile-up of high molecular weight precursor RNA in the nucleus (6, 17), (b) the relatively normal cytoplasmic RNA labeling observed by autoradiography (4), and (c) the more or less normal cytoplasmic RNA content [as correlated to food consumption (2, 3)]. The loss of nucleolar RNA label may be an expression of a system of normal breakdown of RNA (24). Continued analysis of the thioacetamide and the regenerating liver cell should prove useful in uncovering some of the elements involved in ribosome formation.

RESUMEN

Se contrastó el metabolismo del ARN de las células del parénquima hepático en regeneración a diferentes períodos siguientes a la hepatectomía parcial con células hepáticas que responden a la tioacetamida. Se analizaron cuantitativamente los patrones de incorporación de la citidina tritiada en el ARN nucleolar y citoplásmico empleando técnicas autorradiográficas. Se vieron claros desplazamientos en los

patrones de incorporación y transformación a las 6.12 y 20 horas siguientes a la hepatectomía parcial. La incorporación total en el nucleolo de la célula afectada por la tioacetamida mostró un aumento de 25 veces sobre los valores control mientras que el marcado citoplásmico permaneció comparable al nivel del control. Suprimida la droga durante 4 días los animales continuaron mostrando una tasa elevada de incorporación en el ARN nucleolar y un aumento del marcado citoplásmico. Las ratas tratadas con tioacetamida sometidas a hepatectomía parcial mostraron un desplazamiento en los patrones de marcado nucleolar y citoplásmico parecido al de las células hepáticas en regeneración de los animales no tratados. Se presume que la tioacetamida activa la tasa de síntesis del precursor del ARN sin afectar otros procesos involucrados en la formación ribosómica y la transferencia al citoplasma.

REFERENCES

- (1) RATHER, L. J.: Experimental alteration of nuclear and cytoplasmic components of the liver cell with thioacetamide. 1. Early onset and reversibility of volume changes of the nucleolus, nucleus and cytoplasm. *Bull Hopkins Hosp* 88: 38-58, 1951.
- (2) LAIRD, A. K.: Nuclear changes induced in rat liver cells by thioacetamide. *Arch Biochem* 46: 119-127, 1953.
- (3) KLEINFELD, R. G.: Early changes in rat liver and kidney cells induced by thioacetamide. *Cancer Res* 17: 954-962, 1957.
- (4) KOULISH, S., and KLEINFELD, R. G.: The role of the nucleolus. 1. Tritiated cytidine activity in liver parenchymal cells of thioacetamide-treated rats. *J Cell Biol* 23: 39-51, 1964.
- (5) TSUKADA, K., and LIEBERMAN, I.: Metabolism of nucleolar ribonucleic acid after partial hepatectomy. *J Biol Chem* 239: 1564-1568, 1964.
- (6) STEELE, W. J., OKAMURA, N., and BUSCH, H.: Effects of thioacetamide on the composition and biosynthesis of nucleolar and nuclear ribonucleic acid in rat liver. *J Biol Chem* 240: 1742-1749, 1965.
- (7) HIGGINS, G. M., and ANDERSON, R. M.: Experimental pathology of the liver. I. Restoration of the liver of the white rat following partial surgical removal. *Arch Path* 12: 186-202, 1931.
- (8) CHALKLEY, H. W.: Methods for the quantitative morphological analysis of tissues. *J Nat Cancer Inst* 4: 47-53, 1943.
- (9) HARKNESS, R. D.: The spatial distribution of dividing cells in the liver of the rat after partial hepatectomy. *J Physiol (London)* 116: 373-379, 1952.
- (10) KLEINFELD, R. G., and VON HAAM, E.: Nucleic acid metabolism in regenerating rat liver using cytidine- H^3 . *Ann Histochem* 7: 89-96, 1962.
- (11) GRISHAM, J. W.: A morphologic study of deoxyribonucleic acid synthesis and cell proliferation in regenerating rat liver; autoradiography with thymidine- H^3 . *Cancer Res* 22: 842-849, 1962.
- (12) TAYLOR, J. H., and WOODS, P. S.: *In situ* studies of polynucleotide synthesis in nucleolus and chromosomes. In *Subcellular Particles* (Hayashi, T., ed.). New York, Ronald Press Co., 1959, pp 172-185.
- (13) AMANO, M., LEBLOND, C. P., and NADLER, N. J.: Radioautographic analysis of nuclear RNA in mouse cells revealing three pools with different turnover times. *Exp Cell Res* 38: 314-340, 1965.
- (14) KLEINFELD, R. G., and VON HAAM, E.: The effect of thioacetamide on rat liver regeneration. 1. Cytological studies. *Cancer Res* 19: 769-778, 1959.
- (15) BUCHER, N. L. R.: Regeneration of mammalian liver. In *International Review of Cytology* (Bourne, G. H., and Danielli, J. F., eds.). New York and London, Academic Press Inc., 1963, vol 15, pp 245-300.

- (16) STERN, H.: Intracellular regulatory mechanisms in chromosome replication and segregation. *Fed Proc* 22: 1097-1102, 1963.
- (17) PERRY, R. P., and KLEINFELD, R. G.: Unpublished data.
- (18) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (19) ———: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat Cancer Inst Monogr* 14: 73-89, 1964.
- (20) MCCONKEY, E. H., and HOPKINS, J. W.: The relationship of the nucleolus to the synthesis of ribosomal RNA in Hela cells. *Proc Nat Acad Sci USA* 51: 1197-1204, 1964.
- (21) ECKER, R. E.: The role of ribosomal RNA in the control of ribosomal protein synthesis. *Proc Nat Acad Sci USA* 54: 1465-1470, 1965.
- (22) VILLALOBOS, J. G., STEELE, W. J., and BUSCH, H.: Effects of thioacetamide on labeling of ribonucleic acid of isolated nucleoli *in vitro*. *Biochim Biophys Acta* 91: 233-238, 1964.
- (23) ———: Increased ribonuclease activity of liver cells of thioacetamide-treated rats. *Biochem Biophys Res Commun* 17: 723-729, 1964.
- (24) WIESNER, G., ACS, G., REICH, E., and SHAFIQ, A.: Degradation of ribonucleic acid in mouse fibroblasts treated with actinomycin. *J Cell Biol* 27: 47-52, 1965.

DISCUSSION

Busch: One interesting point is that there are some common characteristics between the changes in nucleolar RNA of the regenerating liver and the thioacetamide-treated liver. In each instance there is a remarkable increment in the 35S and 45S RNA in the gradient sedimentation pattern. In the case of the thioacetamide, however, there is a more complicated problem than just increased nucleolar RNA synthesis, *i.e.*, there is apparently an inhibition of the enzyme which we have called "convertase" that transfers or converts 45S and 55S RNA into 35S and 28S RNA, the RNA of the 60S ribonucleoprotein particle. Thioacetamide either blocks the synthesis of this enzyme or inhibits its activity very rapidly. In the regenerating liver there seems to be a change in the feedback which causes an increased synthesis in the regenerating liver.

Kleinfeld: It is of interest to note that the only cells that respond to thioacetamide are the parenchymal cells of the liver and the proximal convoluted tubule cells of the kidney (ref. 3, this paper), both of which function in the detoxification of drugs. It seems likely that a metabolite of thioacetamide formed in these tissues is the effective agent (Nygaard, Eldjarn, and Nakken, *Cancer Res* 14: 625-628, 1954). Apart from the specific effect of the drug on liver and kidney, thioacetamide has been reported to break the dormancy of potatoes, presumably by inactivation of inhibitors of germination (Sexton, ref. 3, this paper). The possibility that thioacetamide, or a metabolite of the drug, interferes with enzymatic reactions which regulate synthesis in the nucleus of liver cells was first suggested by Rather (ref. 3, this paper).

Feinendegen: The renewal data you presented in terms of autoradiographic grain counts over nucleus, nucleolus, and cytoplasm are apt to be influenced considerably by the degree of reutilization of RNA precursors in the living animal. For example, we found that at least 50% of the RNA labeling in rat bone marrow is reutilized (*Ann NY Acad Sci* 113: 727-741, 1964). I guess you would have elaborated on this problem if you had had more time. One needs to take into account first, the potential alterations of the magnitude of the reutilization pathway provoked by injury, such as by thioacetamide, and second, the corresponding effect of these alterations on the renewal measurement.

Autoradiographic, Biochemical, and Ultrastructural Studies Into the Effect of Actinomycin, 5-Fluorouracil, and Adenosine on Nucleolar and Cellular Structure and Function^{1, 2}

UNNE STENRAM, *Department of Pathology, University of Uppsala, Uppsala, Sweden*

SUMMARY

Autoradiographic, biochemical, and ultrastructural studies into the effect of actinomycin, 5-fluorouracil and adenosine on nucleolar and cellular structure and function were made. Actinomycin decreased the nucleolar, and to some extent the cytoplasmic, RNA labeling in the liver of rats, but left the protein labeling unchanged, except possibly in the nucleoli. Ultrastructural changes were pronounced in the nucleoli, limited in other parts of the nuclei, and apparently absent in the cytoplasm. Definite changes were also found in the endoplasmic reticulum of liver cells following renourishing after starvation and liver regeneration after partial hepatectomy, both of which may be thought to imply a

de novo synthesis of liver RNA and protein. In the former, but not in the latter experiment, the actinomycin-treated animals showed less glycogen and lower protein labeling than the controls. Fluorouracil decreased RNA labeling in all parts of liver cells but left the protein labeling essentially uninfluenced. The nucleoli increased in size and displayed ultrastructural changes. Ultrastructural changes were insignificant in other parts of the cells. Adenosine decreased nucleic acid labeling in tissue culture cells but had only a slight effect on the protein labeling. Ultrastructural changes were found in the nucleoli.—*Nat Cancer Inst Monogr* 23: 379–390, 1966.

STUDIES OF intracellular structure and function in this laboratory have been especially concerned with nucleic acids and protein, and nucleoli have therefore figured prominently. During the past few years, substances interfering with normal nucleic acid synthesis have mainly been employed, and since some experience in dealing with the rat liver had already been obtained, this has usually been chosen as the experimental organ. When possible, our animals have been given a protein-free diet, because such a regimen yields large liver nucleoli and a heavy RNA

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² The work was supported by grants from the Swedish Medical Research Council (Projects W 337 and Y 515) and the Swedish Cancer Society (Project 65: 85).

and protein labeling of the liver cell (7, 9, 12). By this means differences in experimental animals may be more easily detected.

I also wish to emphasize that our autoradiographs showing nucleic acid labeling have always been pretreated with ice-cold 5% trichloroacetic acid for 10 minutes to extract acid-soluble nucleotides, although this and other technical details are not reported in every paper.

It is, of course, always essential to ensure that the nutritional conditions are as nearly identical as possible, since the nutritional status of the animals has profound effects on the cytology and biochemistry of the liver cell. Unfortunately, many studies have been reported in which both control and experimental animals were allowed to eat *ad libitum*, even when the substance to be studied, for example, actinomycin, severely interfered with the appetite of the animal and with the digestion and resorption of food.

The enlarged liver cell nucleoli of protein-deprived rats have the same RNA concentration as those of protein-fed animals (8). Feulgen staining gives the same result in liver nucleoli of protein-fed, protein-deprived, and starved rats. The nucleoli often appear faintly Feulgen-positive, which may be due to the highly Feulgen-positive nucleolus-associated chromatin. However, we have also found Feulgen-positive material in the interior of the nucleoli, especially in the large nucleoli of protein-deprived rats (figs. 4-6). Altmann *et al.* (1) found this Feulgen-positive material in the enlarged nucleoli in hepatomas, and ascribed it to the presence of nucleolar DNA. The same interpretation may be valid for the nucleoli described here. The ultrastructure of the liver nucleoli also seems to be essentially the same for these three conditions, when the protein-free diet has been administered for only a few days, except for a concentration of pars amorpha on the periphery of the nucleoli of starved rats (figs. 1-3). However, when the protein-free diet is administered for several weeks, definite changes do appear in the ultrastructure of the nucleoli (2, 15).

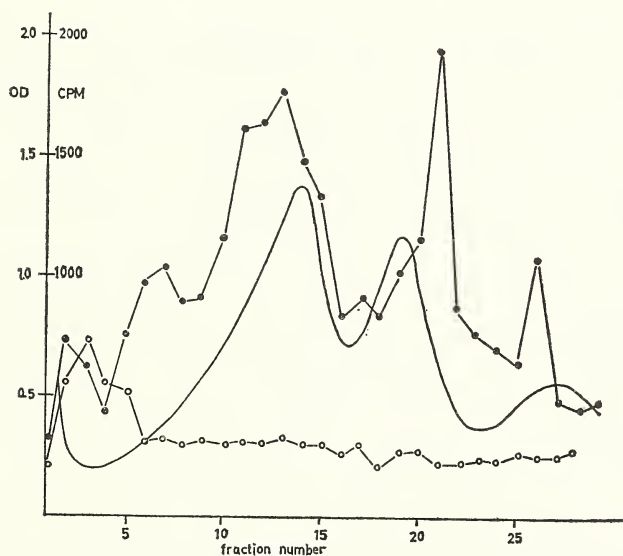
This paper briefly reports on work with three substances which give pronounced ultrastructural changes in the nucleoli, namely, actinomycin, 5-fluorouracil, and adenosine.

Actinomycin decreased the autoradiographic RNA labeling of the liver cell, especially over nucleoli and cytoplasm, but protein labeling was uninfluenced except perhaps in the nucleoli. It brought about a collapse of liver nucleolar structure. Smaller changes also were found in other parts of the nuclei (10). In starving rats, which are known to have a decreasing amount of liver RNA and protein, no structural alterations in the cytoplasm were observed following actinomycin. Thus actinomycin did not seem to induce any increase in the breakdown of cytoplasmic structures containing RNA. In two different experiments, however, it effectively prevented the resynthesis of cytoplasmic structures.

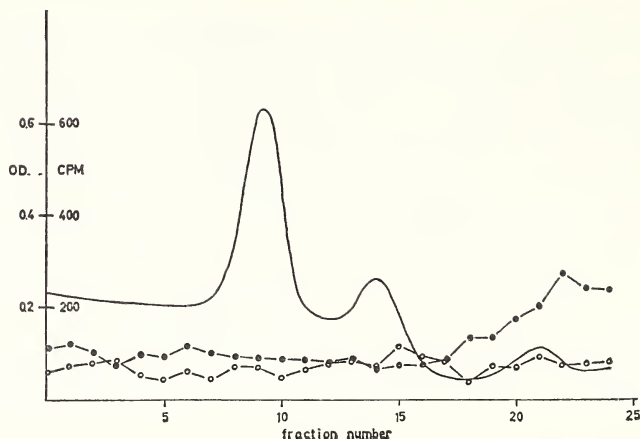
In the first experiment (13) starved animals were renourished by intraperitoneal administration of amino acids and glucose. The control ani-

mals showed a larger amount of smooth-surfaced endoplasmic reticulum and also a larger amount of glycogen in the liver cell cytoplasm than the actinomycin-treated animals. The control rats also showed a higher protein labeling, as determined by autoradiography following the administration of leucine- ^3H . The RNA labeling was higher in the controls also. In the second experiment, structural changes in regenerating liver following two-thirds hepatectomy were very marked. An increased amount of rough-surfaced endoplasmic reticulum was seen in the controls, but not in the actinomycin-treated rats. The controls showed a higher RNA labeling, but there were no differences in the protein labeling (14).

In collaboration with R. Willén, RNA was extracted by the phenol method from the livers of the hepatectomy experiment. Sedimentation patterns in a linear sucrose density gradient showed inhibition of ribosomal RNA synthesis in the actinomycin-treated rats but largely uninfluenced labeling of transfer RNA (text-figs. 1 and 2). The hypertrophy of the ergastoplasm and the rough-surfaced endoplasmic reticulum, as demonstrated here under the optical and electron microscope, respectively, seems thus to require the synthesis of ribosomal RNA.



TEXT-FIGURE 1.—Sedimentation pattern of RNA from livers of partially hepatectomized rats. Extraction with phenol and sodium dodecyl sulfate at pH 5.6 at 60 C. Linear sucrose gradient 5 to 20%, Spinco SW 25.1, 24,000 rpm, 12 hours. Faster-moving components are at left. Curve without circles represents the 2537A absorbance. Curves with open and solid circles represent the cpm radioactivity in liver RNA from 3 animals labeled for 30 minutes and 3 animals labeled for 3 hours with cytidine- ^3H , respectively.



TEXT-FIGURE 2.—Sedimentation pattern of RNA from livers of partially hepatectomized rats treated with actinomycin. For further explanations see text-figure 1. Note absence of labeling of heavy components in the 30-minute animals, and absence of ribosomal RNA labeling in the 3-hour animals.

It has been shown that some inhibitory effects ascribed to actinomycin, such as the prevention of increased uterine protein synthesis after estradiol administration, are in fact due to an increased secretion of adrenocortical hormone elicited by this highly toxic antibiotic. Other agents, such as puromycin, may also have such effects (5). Our hepatectomy experiments were therefore repeated: *a*) with animals given the unphysiologically high dose of 10 mg cortisone acetate per 100 g body weight prior to hepatectomy, and *b*) with gonadectomized and adrenalectomized animals, which were given 150 μ g cortisone acetate and 0.6 mg deoxycorticosterone acetate per animal preoperatively. (The rats in these experiments weighed about 120 g. Adrenalectomized rats which had not received substitution therapy survived for less than 12 hours after the partial hepatectomy.) The results have so far only been analyzed with electron microscopy and optical microscopy. Neither of these two hormonal interferences had any influence on the inhibitory effect of actinomycin on liver regeneration, or on the regeneration in the non-actinomycin-treated rats. The results indicate that the inhibitory effect of actinomycin on the regeneration of the liver cell cytoplasm is due to a direct effect of actinomycin on the liver cell.

We have started similar experiments with another substance, 5-fluorouracil. This is commonly known to interfere with nucleic acid metabolism (4). We administered large doses of it to rats. The RNA labeling was largely suppressed in all parts of the liver cell, while the protein labeling was uninfluenced. The liver cell nucleoli increased in size and displayed ultrastructural changes. Ultrastructural alterations were insignificant in other parts of the cells (11).

It has long been known that adenosine brings about a change in the light microscopic morphology of the nucleoli in tissue culture cells. We tried to produce the same changes in rat liver cells *in vivo* but failed. These investigations have therefore been performed with tissue culture cells. The autoradiographic RNA and protein labeling were examined. We found a markedly decreased nucleic acid labeling over the whole cell, while the protein labeling was influenced much less or not at all (6).

We have also studied the effect of adenosine on tissue culture cells under the electron microscope. These cultures were kindly supplied by Professor T. Wesslén and Dr. H. Diderholm, Department of Virology, Uppsala.

The nucleoli of the control cells (figs. 7 and 8) were rounded and sharply delimited from the nucleoplasm. They had two electron-dense components. The larger appeared to have the characteristics of pars amorpha (*b* in figs. 7 through 11). Within this there were small areas of even greater density (indicated by *open arrows*), probably identical with the nucleolonema, which also often outlined the third nucleolar component, the electron-lucent areas (*c*). The electron-lucent areas were sometimes similar to the nucleoplasm in osmium-fixed specimens and might then be invaginations from it, as we have seen in some sections. However, especially in specimens prefixed in glutaraldehyde prior to OsO_4 , the electron-lucent areas of the nucleoli were as a rule denser than the nucleoplasm, and these parts at least may be regarded as a special nucleolar component. Sparse, dense granules were sometimes seen in this component (fig. 8).

Treatment with adenosine gave a dissociation of the nucleolar structures (figs. 9 through 11), which, however, occupied a conspicuously large area of the nucleus. A coiled, bandlike component appeared (indicated by *arrows*) with granules, which seemed to be similar to the so-called large granules of the pars amorpha and which sometimes appeared in clusters. This component seemed to arise from, or in close association with, the nucleolonema-like component. As a rule the pars amorpha, the electron-lucent areas, and parts of the nucleolonema-like component could still be traced. After 24 hours the nucleoli appeared almost normal, and there was an increased amount of perichromatin granules.

There were no definite changes in the extranucleolar part of the nucleus. Mitochondria, the endoplasmic reticulum, and cytoplasmic ribosomes and polysomes seemed to be unchanged during adenosine treatment.

The three substances discussed in this paper have the property in common of largely suppressing the RNA labeling of the cells, without influencing the protein labeling to any appreciable extent. In those cells which have not been forced by the experimental conditions to perform a *de novo* synthesis of protein, the synthesis of protein does not seem to have been influenced at all, except perhaps in the nucleoli. This holds for the experiments with starving rats. In the other experiments with rats and in the actively growing tissue culture cells, there may be a stimulus for a new synthesis of protein, and here suppression was seen in some of the

experiments. In regard to the morphology, the most pronounced changes were found in the nucleoli; this points to the crucial role of the nucleolus in RNA synthesis. The protein-synthesizing complex of the cytoplasm (polysomes, ribosomes, endoplasmic reticulum) appeared to be comparatively stable during these conditions, continuing to synthesize protein for several hours though the supply of newly synthesized RNA seemed to be heavily suppressed.

Several authors have discussed the possibility that the cytoplasmic ribosomes may be synthesized in the nucleolus. This now seems less probable to us. First, the nucleolar "granules" are morphologically very similar to the cytoplasmic ribosomes but do not seem to be identical. Second, cytoplasmic ribosomes consist of about equal amounts of RNA and protein. The nucleoli have a high RNA turnover but a low protein turnover. The protein part of the cytoplasmic ribosomes may therefore not derive from the nucleolus, but perhaps from the cytoplasm. Third, evidence has been presented indicating that preribosomal components appear in the cytoplasm (3). Our observations are, however, perfectly compatible with the view that the RNA of the cytoplasmic ribosomes derives from the nucleolus.

Note added in proof: It was evident during the Symposium that there was no unanimity on the use of the term "nucleolonema." In this paper and in our previous papers, the nucleolonema has been used to denote the fibrillar part of the electron-dense areas of the nucleoli. Pars amorpha has been used to denote the granule-rich part of the electron-dense areas of the nucleoli. There is as a rule a gradual transition between these two parts.

RESUMEN

Estudios radioautográficos, bioquímicos y ultraestructurales sobre el efecto de la actinomicina, 5-fluorouracilo y adenosina sobre la estructura y la función nucleolar y celular. La actinomicina disminuyó la marcación del ARN nucleolar y, en alguna extensión, la citoplásmica en los hígados de ratas, pero no cambió la marcación de las proteínas excepto quizá en el nucleolo. En los nucleolos, los cambios ultraestructurales fueron pronunciados, limitados en otras partes de los núcleos y, aparentemente, ausentes en el citoplasma. Se hallaron también cambios definidos en la estructura citoplásmica en dos diferentes experimentos, de los cuales puede pensarse que implica una síntesis *de novo* del ARN y de las proteínas hepáticas, especialmente en la realimentación después del ayuno, y en la regeneración hepática después de una hepatectomía parcial. En ambos experimentos se hallaron cambios en el retículo endoplásmico. En el primero, pero no en el último experimento, los animales tratados con actinomicina mostraron menos glucógeno y una menor marcación proteica que los controles.

El fluorouracilo disminuyó la marcación del ARN hepático en todas las partes de la célula pero, en esencia, no influyó en la marcación proteica. Los nucleolos del hígado aumentaron de tamaño y exhibieron cambios ultraestructurales. Los cambios ultraestructurales no fueron de significación en otras partes de las células.

La adenosina disminuyó la marcación del ácido nucleico en células de cultivo de tejidos, pero sólo tuvo un leve efecto en la marcación proteica. Se hallaron cambios ultraestructurales en los nucleolos.

REFERENCES

- (1) ALTMANN, H.-W., STÖCKER, E., and THOENES, W.: Über Chromatin und DNS-Synthese im Nucleolus. *Z Zellforsch* 59: 116-133, 1963.
- (2) DAVID, H.: Physiologische und pathologische Modifikationen der submikroskopischen Kernstruktur. III. Der Nucleolus. *Z Mikr Anat Forsch* 71: 551-586, 1964.
- (3) GIRAUD, M., LATHAM, H., PENMAN, S., and DARNELL, J. E.: Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm, *J Molec Biol* 11: 187-201, 1965.
- (4) HEIDELBERGER, C.: Biochemistry of 5-fluorouracil. *In* *Chemotherapy of Cancer* (Plattner, P., ed.). Amsterdam, London and New York, Elsevier, 1964, pp 88-98.
- (5) LIPPE, B., and SZEGO, C.: Participation of adrenocortical hyperactivity in the suppressive effect of systemic actinomycin D on uterine stimulation by oestrogen. *Nature (London)* 207: 272-274, 1965.
- (6) STENRAM, I., RYBERG, H., and STENRAM, U.: Radioautographic examination of nucleic acid and protein labelling of adenosine-treated tissue culture cells. *Acta Path Microbiol Scand* 64: 289-293, 1965.
- (7) STENRAM, U.: Nucleolar size in the liver of rats fed on high and nonprotein diets after starvation. *Acta Anat (Basel)* 26: 350-359, 1956.
- (8) ———: Interferometric determinations of the ribose nucleic acid concentration in liver nucleoli of protein-fed and protein-deprived rats. *Exp Cell Res* 15: 174-183, 1958.
- (9) ———: Radioautographic studies with methionine-³H and cytidine-³H in protein-fed, protein-deprived and starved rats. *Z Zellforsch* 58: 107-124, 1962.
- (10) ———: Electron-microscopic study on liver cells of rats treated with actinomycin D. *Z Zellforsch* 65: 211-219, 1965.
- (11) ———: Cytological, radioautographic and ultrastructural studies on the effect of 5-fluorouracil on rat liver. *Z Zellforsch* 71: 207-216, 1966.
- (12) STENRAM, U., and HIRSCHMAN, R.: Radioautographic studies of the protein synthesis in rat during protein deprivation and starvation. *Acta Anat (Basel)* 61: 445-453, 1965.
- (13) STENRAM, U., VANNEFÄLT, J., and WILLÉN, R.: Cytological, radioautographic and ultrastructural studies on the effect of actinomycin on the liver of renourished rats. *Z Zellforsch* 66: 854-866, 1965.
- (14) STENRAM, U., and WILLÉN, R.: The effect of actinomycin D on ultrastructure and radioautographic ribonucleic acid and protein labeling in rat liver after partial hepatectomy. *Cancer Res* 26: 765-772, 1966.
- (15) SVOBODA, D., and HIGGINSON, J.: Ultrastructural changes produced by protein and related deficiencies in the rat liver. *Amer J Path* 45: 353-379, 1964.

PLATE 48

FIGURES 1-3.—Electron micrographs of liver cell nucleoli. The nucleoli in figures 1 and 2 are very similar. In figure 3 the pars amorpha is mainly situated in the periphery of the nucleolus. OsO_4 fixation. Uranyl acetate-lead citrate staining. $\times 35,000$

FIGURES 4-6.—Liver cell nuclei. Fixation in Carnoy's fluid No. II. Feulgen staining. Note Feulgen-positive material in the interior of the nucleoli. $\times 3,100$

FIGURES 1 AND 4.—From a group of male rats weighing 45 g, which were starved for 1 day and then fed on a protein-free diet (7) for 5 days. During this period the rats decreased 10 g in weight.

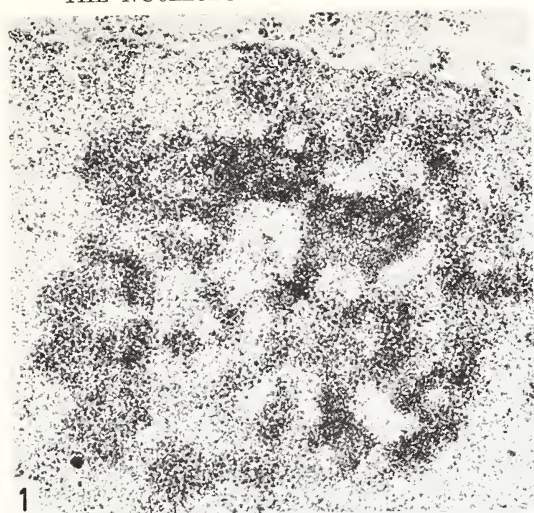
FIGURES 2 AND 5.—From a group of male rats weighing 42 g, which were fed on a protein-rich diet (7) for 5 days. During this period they increased 19 g in weight.

FIGURES 3 AND 6.—From a group of male rats weighing 45 g, which were starved for 3 days. During this period the rats decreased 13 g in weight.

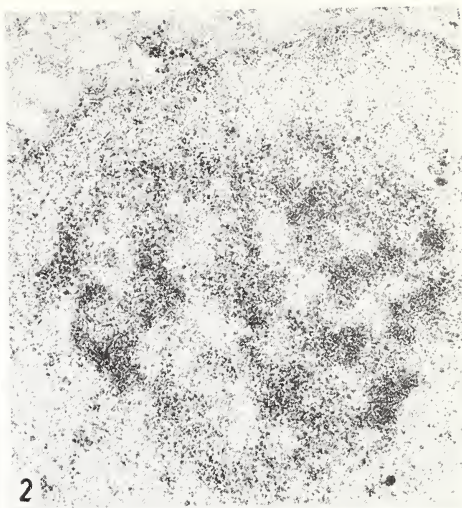
FIGURES 7 AND 8.—Electron micrographs of nucleoli of monkey kidney cells in tissue culture. Two electron-dense components (*b* corresponding to pars amorpha and *open arrows* corresponding to the nucleolonema) and 1 or 2 electron-lucent components (*c*) are seen. Controls to figures 9-11. $\times 26,000$

FIGURE 7.—Fixation in 5% glutaraldehyde 5 hours, postfixation in OsO_4 2 hours. Uranyl acetate-lead citrate staining.

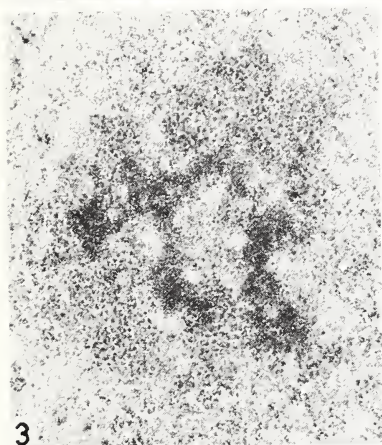
FIGURE 8.—Fixation in OsO_4 2 hours. Uranyl acetate staining.



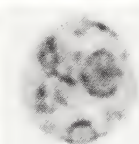
1



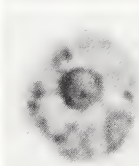
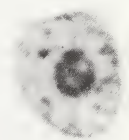
2



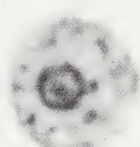
3



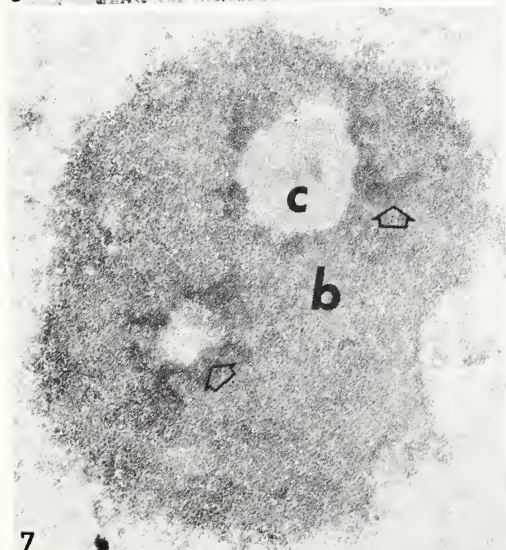
4



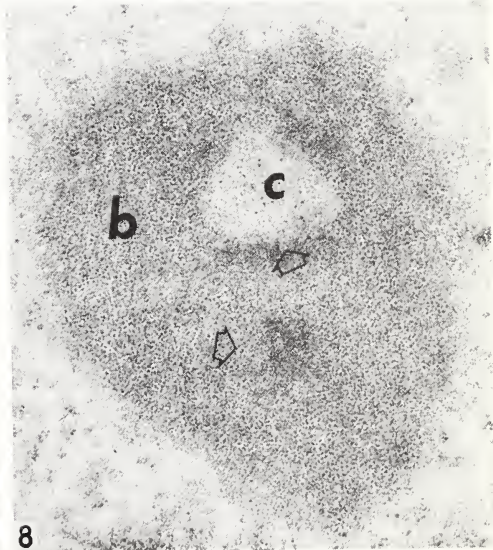
5



6



7



8

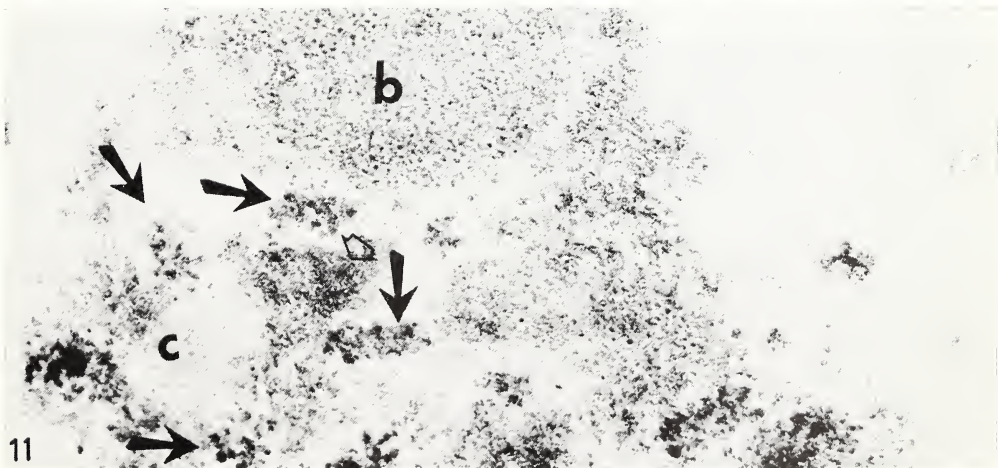
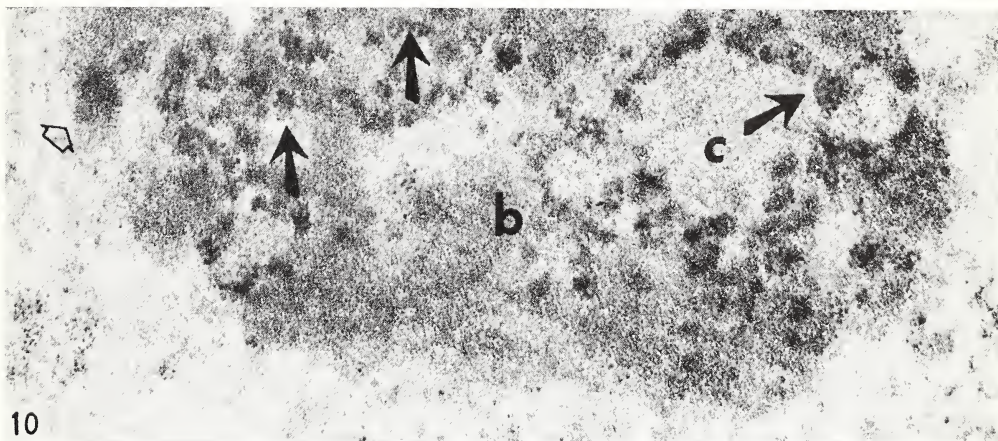
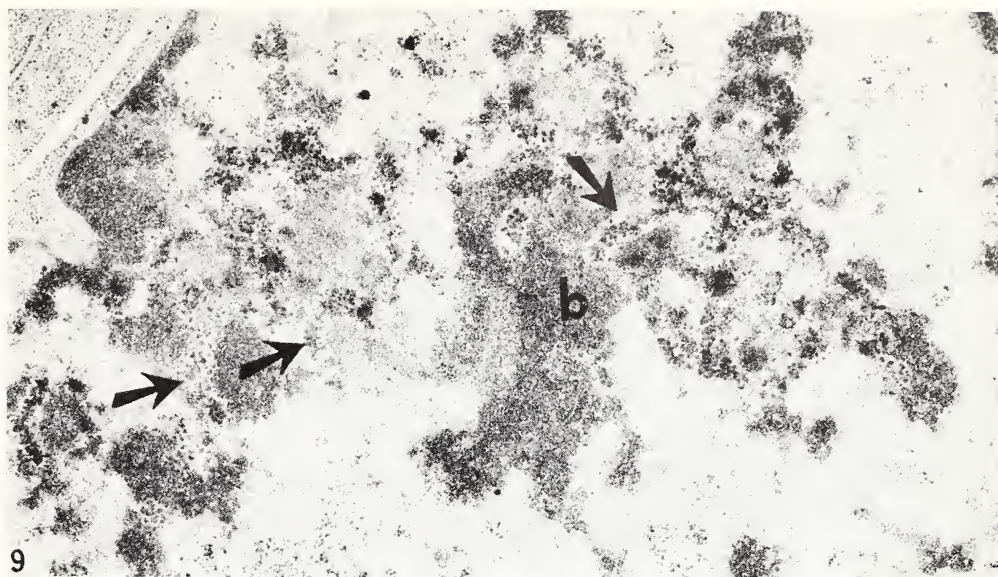
PLATE 49

FIGURES 9-11.—Electron micrographs of nucleoli of monkey kidney cells in tissue culture, treated with 2 mM adenosine for 6 hours. The nucleoli are more or less dissociated. *Arrows* point to parts of a bandlike component. For further explanation *see* figures 7 and 8. Uranyl acetate staining.

FIGURE 9.—Fixation in 5% glutaraldehyde 5 hours, post fixation in OsO_4 2 hours. $\times 26,000$

FIGURE 10.—Fixation in OsO_4 2 hours. $\times 26,000$

FIGURE 11.—Details of a nucleolus showing the granules in the bandlike component. The same fixation as in figure 9. $\times 51,000$



DISCUSSION

Mandel: We have an explanation for the increase of the incorporation of radioactive precursors into cellular RNA during protein starvation. We found that under these conditions the number of polysomes as well as the quantity of ribosomes decreases (Life Sci, in press) and that the turnover of ribosomal RNA (rRNA) and messenger RNA (mRNA) increases in parallel with a higher activity of the RNA polymerase [Mandel, Bull Soc Chim Biol (Paris) 46: 43-70, 1964]. So it appears that a faster degradation of mRNA and rRNA produces a derepression of RNA synthesis. This faster degradation is due to the fact that in the absence of the input of essential amino acids, protein synthesis decreases and the formation of ribosomal particles and fixation of mRNA on the ribosomes, which protects the RNA against ribonuclease, is disturbed.

Waddington: I want to draw attention to the fact that actinomycin seems to produce very different types of structural effects in different sorts of cells. In particular, Dr. K. Jones in our laboratory in Edinburgh has detected some very striking ultrastructural effects of actinomycin, which are probably associated with the nucleoli, in certain amphibian embryonic cells, particularly of *Rana* (J Cell Biol 21: 245-252, 1964; Devel Biol, in press). In cultured cells from certain tissues, he finds that the application of actinomycin brings about the appearance within the nuclei of bundles of relatively coarse threads. The threads lie parallel to one another for lengths at least up to 1 μ , but the bundles may be flexed relatively sharply so that a single section may cut one part transversely to the threads, and another part more or less parallel to them. Bundles may contain up to 200 threads. In transverse section they are arranged in an orderly hexagonal pattern, each thread having a thickness of about 250 Å. Several aggregates may be seen in a single section of a nucleus, but they seem to be most common in the neighborhood of the nucleolus. They have never been found in pregastrula cells in which a nucleolus has not yet formed, nor have they been seen in certain adult tissues, such as kidney and heart muscle, in which the nucleoli are poorly developed and stain weakly. On the other hand, they appear in rapidly growing embryonic cells characterized by strongly staining nucleoli. The evidence for a definite association with the nucleolus is, however, not yet complete. They are remarkable in being some of the most definite structural formations detected within nuclei in general by electron microscopy.

Stenram: I have looked for similar changes in liver cells but did not find any.

Taylor: Does anyone have ideas concerning the effects of adenosine on metabolism which would produce its effect?

Lettré: We came to the conclusion that quite probably adenosine in this unphysiologically high concentration is acting by mediating the solubility of the nucleolar material, thus releasing the material in the nuclear sap and unmasking the structures to which the material was adhering. This is the interpretation of organic chemists who use adenosine to enhance the solubility of substances which have a low solubility in aqueous media.

NUCLEOLAR GENES

Is the nucleolus the product of a single genetic locus? This is a problem that has intrigued cytologists since the discovery of the *nucleolus organizer* more than 30 years ago. The relationship of *the nucleolus* to secondary constructions, the "puffs" of Diptera, and other nucleolus-like bodies has been explored and debated in the intervening years. The discovery of the "Oxford factor," an anucleolate mutation in the frog, *Xenopus laevis*, by Fischberg several years ago has provided new material and impetus for the study of this question. In this series of papers, there is the very elegant demonstration of a specific gene product of the nucleolus organizer region, although the problem of whether the nucleolus is produced by a single genetic locus is still not entirely settled. Appropriately, this session was chaired by Dr. Barbara McClintock and Dr. C. Pavan, both pioneers in the analysis of genetic control of the nucleolus.

Diversity and Variation of the Nucleolar Organizing Regions in Chironomids¹

C. PELLING and W. BEERMANN, *Max-Planck-Institut für Biologie, Tübingen, Germany*

SUMMARY

Our studies on ordinary and accessory nucleoli of giant chromosomes have demonstrated that the nucleolus is a highly specialized "puff": 1) Nucleoli originate in cytologically well-defined bands of the giant chromosomes comprising less than 1/200 of the total genome. 2) Usually RNA synthesis in nucleoli occurs close to the chromosome. 3) RNA synthesis in nucleoli is regulated independently of that in other loci. 4) Typically, all nucleolar organizers of the *Chironomus* genome are interchange-

able. Organizers with deficient functions may, however, occur. 5) One band which forms a nucleolus has been fragmented into two functionally intact portions, indicating that the nucleolar organizer consists of multiple genetic subunits. 6) Loss of the nucleolar organizers begins to interfere with development at the time of gastrulation. Further development is characterized by a general disturbance of growth and organization, but not of cellular differentiation.—*Nat Cancer Inst Monogr* 23: 393–409, 1966.

AMONG THE hundreds of gene loci whose activity becomes cytologically manifest within a salivary gland nucleus, the nucleolar organizing regions constitute a special class. It is true that at a first glance the nucleolus does not appear to possess any of the typical features of a puff. Usually it is not seen to derive from a very small chromosomal segment equivalent to one DNA band in giant chromosomes. Furthermore, the amount of ribonucleoprotein present in the nucleolus and its activity in RNA production are so tremendous that for a long period the nucleolus has been interpreted as a place of RNA accumulation rather than RNA synthesis. Finally, the nucleolus does not exhibit the variable behavior so characteristic of most puffs. In all tissues that can generally be studied, the nucleolar organizer is active in producing a nucleolus of the same shape and relative dimension. Nevertheless, it can be shown that the nucleolar organizer in principle behaves like other active loci, at least in giant chromosomes.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

MORPHOLOGICAL APPEARANCE OF NUCLEOLAR ORGANIZING REGIONS IN GIANT CHROMOSOMES

A functional nucleolus never seems to be formed without a nucleolar organizer. Generally, the structural condition of giant chromosomal nucleoli makes it difficult to localize exactly the chromosomal region to which the nucleolus is attached. This place is split in both directions along the chromosomal axis and exhibits almost complete disorganization of the adjacent banding pattern. The segment where the nucleolus is attached to the salivary gland chromosome of *Acricotopus lucidus* is a good example (fig. 1) (1). On the other hand, more favorable conditions may be met with in some exceptional material such as the chromosomes in the malpighian tubules in adults of *Chironomus tentans* (2). In these adults the nucleoli have remarkably decreased in size (fig. 2). Similarly, in prepupae of *Acricotopus lucidus* the salivary gland nucleoli gradually regress and finally reach the size and the appearance of a regular puff (fig. 3). In such preparations the nucleolus is attached to a chromosomal segment which looks like a single, or more often, a double band, and which as a band is somewhat thicker than average. In cytological terms this segment can be interpreted as euchromatic, for even in its most condensed condition it appears less Feulgen-positive than most other bands. This may be due to some residual puffing. Puffing, in a very general sense, has, of course, been known to occur in nucleolar organizing regions of giant chromosomes for a long time (1). The regions usually appear split up into many fine strands, sometimes in the manner of a network (3). However, it could be argued that puffing in this case does not really involve the organizer segment itself. Some observations can be brought forward against this view. In *Chironomus tentans* which has two nucleolar organizing regions in its genome, both organizers have sometimes been found in an exceptional condition. In this instance they assume the structure of another highly specialized puff, a Balbiani ring (4). The nucleolar material no longer forms a uniform nucleolus; several droplets and spheres of nucleolar ribonucleoprotein are seen attached loosely to the periphery of the nucleolar Balbiani ring (fig. 4). This is highly unusual because the nucleolar organizer in an active nucleolus will not normally emerge visibly from the surrounding ribonucleoprotein mass. Usually, therefore, the chromosomal elements organizing the nucleolus, and those immediately adjacent to it, can be demonstrated only by means of sensitive staining methods, such as acridine orange, or autoradiography. Radioactive thymidine administered for several hours labels the DNA of some reduplicating nuclei. DNA label extends into the nucleolus, presumably as a reflection of puffing of the organizer (fig. 5). In some cases one can detect a complete permeation of large nucleoli by DNA material (5) opposite to the situation found in malpighian tubules. In different individuals, all transitional stages of nucleolar puffing may be found between

the two extremes, also without necessarily concomitant changes in the amount of nucleolar material. This indicates that nucleolar puffing is not just a mechanical consequence of the accumulation of nucleolar material. If we regard the nucleolar organizer as a dynamic structure, the more interesting question is whether the morphological changes occurring in the nucleolar organizing segments are correlated with changes in nucleolar activity. Such a relationship is indeed suggested by the fact that condensed organizers appear almost exclusively under conditions when metabolic activity is at a minimum, *e.g.*, when salivary glands stop growing prior to cell lysis, or under conditions of starvation. In rapidly growing *Chironomus* larvae, on the other hand, especially those of *Chironomus thummi*, the nucleolar organizing regions are most frequently found in an extremely puffed condition. More direct insight into the variation of nucleolar activity can be gained by autoradiography after uptake of H^3 -uridine. We found that the area of the nucleolus which incorporates uridine into high molecular RNA undergoes interesting changes (5). In *Chironomus tentans* early nucleolar incorporation most frequently takes place close to the chromosome (fig. 6). With increasing incorporation times the label then gradually appears in the periphery of the nucleolus (fig. 7). The uptake of uridine is not always initially limited to the chromosomal neighborhood. It can appear simultaneously throughout the entire nucleolus. Apparently these variations cannot simply be correlated with the rate of uridine incorporation and the size of the nucleolus, as one might expect according to the experience with typical puffs. However, in those cells in which the uridine uptake of the nucleolus is definitely lower than the level of incorporation in Balbiani rings and that in other large puffs, the label is always restricted to the immediate neighborhood of the chromosome (fig. 8). This suggests that the structural state of the nucleolar organizing segment is a factor in the regulation of nucleolar RNA synthesis. The reverse situation is less clear. Nucleolar activity, which exceeds the activity of other chromosomal loci, is not necessarily correlated with maximum puffing of the organizer. Rather high levels of labeling may also occur when the incorporation is spatially restricted to the core of the nucleolus.

Density gradient studies of the newly synthesized RNA, in which we can correlate the autoradiographic pattern of synthesis with the peak pattern of different RNA types, may throw more light on the molecular basis of the morphological changes just described. At present we can only say that there is indeed a lot of variation in the density gradient patterns, when animals with different autoradiographic incorporation patterns are compared. We find differences particularly in the relative heights of individual peaks, including those so far presumed to be of nucleolar origin, such as the $> 28S$ peaks (6-8).

Specific regulation of nucleolar activity may also be correlated with cell differentiation: A special nucleolar organizing region in *Acricotopus*

lucidus behaves differently in the two portions of the salivary glands (9) which produce different types of secretion (1). Within the anterior lobe of the gland the nucleolar organizing segment develops a typical large nucleolus, whereas the same region completely fails to puff within the cells of the main and the accessory lobe, and no nucleolar material accumulates.

The behavior of the active center within the nucleolus has been presented in detail to show that functional interpretations based on only one of the various states of the organizer must be regarded with caution. Thus, under the assumption that the organizer region never extends into the periphery of the nucleolus, differences in the incorporation pattern have been regarded as indicating two separate nucleolar incorporation systems (10-12). The second kind of nucleolar RNA synthesis was thought to be concerned with the production of 4S RNA and to function independently of the organizer chromatin. However, the differential effect of antimetabolites such as benzamide and substituted benzimidazoles must not necessarily be interpreted as demonstrating the existence of two independent RNA synthesizing systems of different nature. In *Chironomus tentans*, for instance, we found that at a certain concentration of actinomycin D the RNA synthesis in one small Balbiani ring is always less affected than that in two other large ones. There is no doubt, however, that in all three of these puffs RNA synthesis is DNA-dependent (17). Moreover, as regards the possible production of 4S RNA in the nucleolus (13), it must be kept in mind that synthesis of 4S RNA has proved to be DNA-dependent (14-15), and anucleolate mutants of *Xenopus* continue to synthesize 4S RNA (16).

DIFFERENT NUCLEOLAR ORGANIZERS IN THE *CHIRONOMUS* GENOME

The genome of *Chironomus tentans* normally contains one nucleolar organizer on the second and one on the third chromosome. Occasionally a third one occurs on the end of the fourth chromosome, the accessory nucleolus. The latter easily can be distinguished from both of the others by its regular spherical outline and by a lighter RNA staining in fixed preparations. It tends to associate with the ordinary nucleoli, but the material of the two different nucleoli remains separate (fig. 9).

If one hybridizes *Chironomus tentans* with *Chironomus pallidivittatus*, a related species with only one nucleolar organizer, the hybrids are heterozygous for the three nucleolar organizing regions, because the nucleolus in *Chironomus pallidivittatus* is formed at a site which is not homologous with either of the two nucleolar organizers in *Chironomus tentans*. In the offspring of such hybrids one finds that all combinations of nucleolar organizing regions are viable. That is to say each organizer can replace all others. The particular combination does not play a role

as long as at least one heterozygous nucleolar organizing region is present in the genome (2). The equivalence of the two regular nucleolar organizers in *Chironomus tentans* is also shown by autoradiographical observations. They are always equal in their level of uridine incorporation, and they invariably show the same degree of puffing (5).

A striking fact from our cytogenetic studies on *Chironomus* is that even fragments of the nucleolar organizer can replace the functions of a complete one (2). In two experiments, X-ray treatment led to the breakage of the organizers into two subunits, each of which was capable of producing a nucleolus. In one of the mutant strains which could be tested in further breeding experiments, the relation in size between the two resulting partial organizers was about 1 to 4. The smaller fragment always developed a correspondingly smaller nucleolus when it was present together with the larger one in the same nucleus (fig. 10). When the two partial organizers are separated, each can carry out the functions of a complete organizer. Thus, one concludes that there is complete homology not only between different nucleolar organizing regions within a chromosome complement, with the exception of accessory organizers, *see below*, but also between subunits of each organizer itself. These findings agree with recent hybridization experiments, especially the work of Ritossa and Spiegelman (18) and Wallace and Birnstiel (19).

Embryos developing from fertilized eggs without any nucleolar organizer do not survive. Anucleolate zygotes also fail to survive if the organizing region of an accessory nucleolus is introduced into the anucleolate genome. This is so, probably because the accessory organizer synthesizes too little RNA and/or RNA of a mutant type. In autoradiographs at least (5) it is evident that its synthetic capacity is far below the level of the ordinary organizing region. Apparently the organizer of the accessory nucleolus is deficient in some way without having lost the property to accumulate ribonucleoproteins. This idea becomes fully plausible when it is recognized that a group of related *Chironomus* species (*Chironomus thummi*) has the regular nucleolar organizer on the fourth chromosome in a position which possibly is homologous to the band where the accessory nucleolus is formed in *Chironomus tentans*.

Some final remarks are devoted to the manner in which the development of anucleolate embryos of *Chironomus* deviates from normal. Blastoderm is always formed in the same way and at the same time as in nucleolate zygotes. Beginning with gastrulation, however, we find increasing signs of developmental aberrations which can in principle be described as being due to a failure to grow and use up the yolk. More or less severe disorganization occurs, although all three germ layers may be formed and some of the larval organs may develop. A complete larva is never formed (fig. 11). It is significant that normal differentiation of many known cell types occurs in these embryos, such as muscle cells, nerve cells, and larval eye pigment cells. Cytological differences between anucleolate and nucleolate embryos also occur as soon as the blastoderm is being

formed. At this stage, large nucleoli become visible for the first time in normal embryos, whereas the deficient cells exhibit numerous small intranuclear bodies of a characteristic spherical outline (fig. 12). A fundamentally similar unspecific failure of development also has been reported to occur in the anucleolate mutant of *Xenopus laevis* (20). The only obvious difference seems to be that in *Chironomus* breakdown of development begins earlier and in a much more pronounced manner than in the frog. This might be due to the special type of yolk utilization in insects which depends on the activity of specialized cells, the vitellophages. A decline of metabolic functions of this cell type because of lack of ribosomes must amplify the consequences of the nucleolar deficiency for all other embryonic cells.

RESUMEN

Nuestros estudios sobre los nucleolos comunes y los nucleolos accesorios de los cromosomas gigantes han demostrado que el nucleolo es un "puff" altamente especializado (sitio cromosómico de síntesis del ARN) :

1) Los nucleolos se originan en bandas citológicamente bien definidas de los cromosomas gigantes comprendiendo alrededor de 1/500 del genomio total.

2) Por lo general, la síntesis del ARN se produce en regiones nucleolares cercanas al cromosoma.

3) La síntesis del ARN en los nucleolos se regula independientemente de la de otros loci.

4) Todos los organizadores nucleolares del genomio del *Chironomus* son típicamente intercambiables. Pueden aparecer, sin embargo, organizadores con funciones deficientes.

5) Se ha fragmentado una banda que forma un nucleolo en dos porciones funcionalmente intactas, lo que indica que el organizador nucleolar se compone de múltiples subunidades genéticas.

6) La pérdida de los organizadores nucleolares comienza a interferir con el desarrollo en el momento de la gastrulación. El desarrollo ulterior se caracteriza por un disturbio general del crecimiento y de la organización, pero no de la diferenciación celular.

REFERENCES

- (1) MECHELKE, F.: Reversible Strukturmodifikationen der Speicheldrüsen-chromosomen von *Acricotopus lucidus*. *Chromosoma* 5: 511-543, 1953.
- (2) BEERMANN, W.: Der Nukleolus als lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.
- (3) POULSON, D. F., and METZ, C. W.: Studies on the structure of nucleolus forming regions and related structures in the giant salivary gland chromosomes of Diptera. *J Morph* 63: 363-395, 1938.
- (4) BEERMANN, W.: Chromomerenkonstanz und spezifische Modifikationen der Chromosomenstruktur in der Entwicklung und Organdifferenzierung von *Chironomus tentans*. *Chromosoma* 5: 139-198, 1952.
- (5) PELLING, C.: Ribonukleinsäure-Synthese der Riesenchromosomen. Autoradiographische Untersuchungen an *Chironomus tentans*. *Chromosoma* 15: 71-122, 1964.
- (6) PERRY, R. P.: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat Cancer Inst Monogr* 14: 73-89, 1964.

- (7) PERRY, R. P., and SRINIVASAN, P. R., and KELLEY, D. E.: Hybridization of rapidly labeled nuclear ribonucleic acids. *Science* 145: 505-507, 1964.
- (8) STEELE, W. J., OKAMURA, N., and BUSCH, H.: Effects of thioacetamide on the composition and biosynthesis of nucleolar and nuclear ribonucleic acid in rat liver. *J Biol Chem* 240: 1742-1749, 1965.
- (9) PANTITZ, R.: Gewebespezifische Manifestierung einer Heterozygotie des Nucleolus in Speicheldrüsenchromosomen von *Acricotopus lucidus* (Chironomide). *Naturwissenschaften* 47: 359, 1960.
- (10) SIRLIN, J. L., and JACOB, J.: Sequential and reversible inhibition of synthesis of ribonucleic acid in the nucleolus and chromosomes: Effect of benzamide and substituted benzamidazoles in Dipteran salivary glands. *Nature (London)* 204: 545-547, 1964.
- (11) BIRNSTIEL, M. L., JACOB, J., and SIRLIN, J. L.: Analysis of nucleolar RNA synthesis in Dipteran salivary glands. Symposium molecular cytology. *Arch Biol.* In press.
- (12) BIRNSTIEL, M. L., SIRLIN, J. L., and JACOB, J.: The nucleolus: a site of transfer ribonucleic acid synthesis. *Biochem J* 94: 10-11, 1965.
- (13) SIRLIN, J. L., JACOB, J., and BIRNSTIEL, M. L.: Synthesis of transfer RNA in the nucleolus of *Smittia*. *Nat Cancer Inst Monogr* 23: 255-270, 1966.
- (14) GOODMAN, H. M., and RICH, A.: Formation of a DNA-soluble RNA hybrid and its relation to the origin, evolution, and degeneracy of soluble RNA. *Proc Nat Acad Sci USA* 48: 2101-2109, 1962.
- (15) RITOSSA, F. M., ATWOOD, K. C., LINDSLEY, D. L., and SPIEGELMAN, S.: On the chromosomal distribution of DNA complementary to ribosomal and soluble RNA. *Nat Cancer Inst Monogr* 23: 449-472, 1966.
- (16) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (17) BEERMANN, W.: In preparation.
- (18) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (19) WALLACE, H., and BIRNSTIEL, M. L.: Ribosomal cistrons and the nucleolus organizer. *Biochim Biophys Acta* 114: 296-310, 1966.
- (20) ELSDALE, T. R., FISCHBERG, M., and SMITH, S.: A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp Cell Res* 14: 642-643, 1958.

PLATE 50

FIGURE 1.—Nucleolus-bearing chromosome segment of *Aericotopus lucidus* deriving from the main lobe of a larval salivary gland. From Mechelke (1).

FIGURE 2.—Heterozygous band carrying the nucleolus (N). Malpighian tubules from a hybrid of *Chironomus tentans* \times *Chironomus pallidivittatus*. From Beermann (2).

FIGURE 3.—Reduced nucleolar organizing region (between band 52 and 53). Main lobe of the salivary gland. Prepupa of *Aericotopus lucidus*. From Mechelke (1).

FIGURE 4.—Formation of a Balbiani ring at the nucleolar organizing region of the third chromosome. *Chironomus tentans*. From Beermann (4).

FIGURE 5.—DNA distribution within the nucleolus. Salivary gland of *Chironomus thummi thummi* \times *Chironomus thummi piger* 7 hours after application of tritiated thymidine. Autoradiograph. Original.

FIGURE 6.—Distribution of nucleolar RNA label. Nucleolus is attached to the second and third chromosome. Incubation time 10 minutes. Salivary gland nucleus of *Chironomus tentans*. Autoradiograph. From Pelling (5).

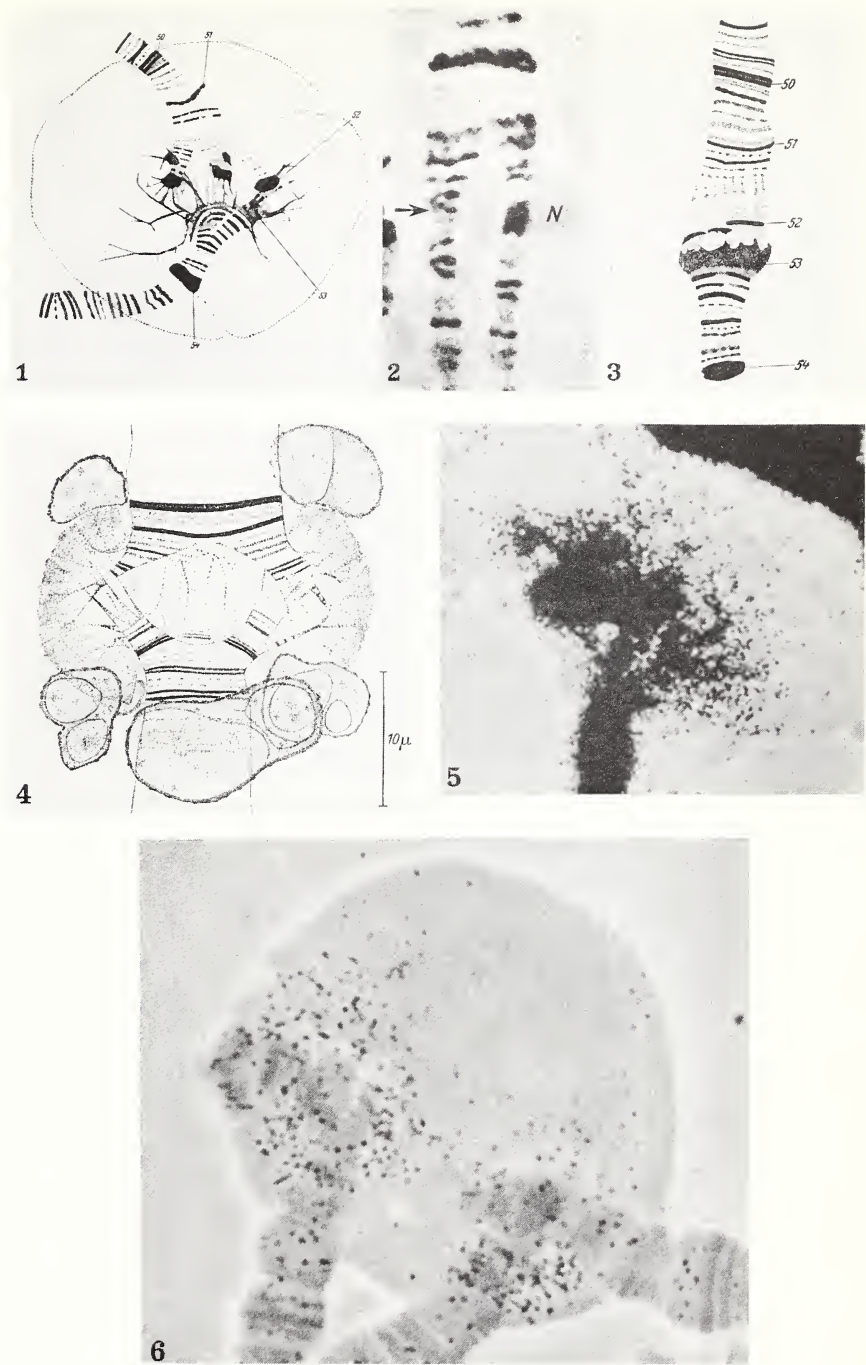


PLATE 51

FIGURE 7.—Distribution of RNA label within the nucleolus of the second chromosome. Incorporation time 30 minutes. Salivary gland nucleus of *Chironomus tentans*. Autoradiograph. Original.

FIGURE 8.—Reduced RNA synthesis at the nucleolus attachment point of chromosome 2 (*arrow*). Salivary gland of *Chironomus tentans* 30 minutes after application of tritiated uridine. Autoradiograph. From Pelling (5).

FIGURE 9.—Accessory nucleolus which forms an aggregate with a regular nucleolus. Salivary gland of *Chironomus pallidivittatus*. From Beermann (2).

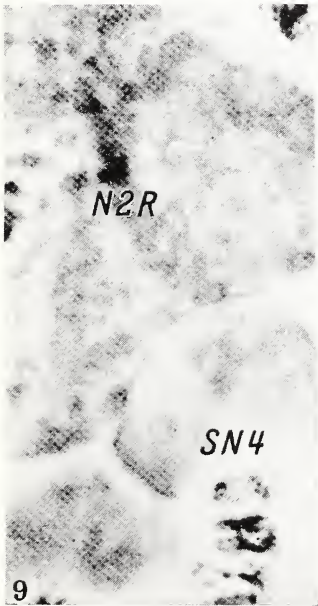
FIGURE 10.—Malpighian tubule nucleus with two fragments of the nucleolar organizing band. *Chironomus pallidivittatus*. For cytological details *see* (2). From Beermann (2).



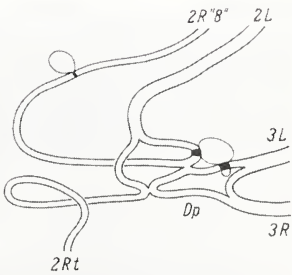
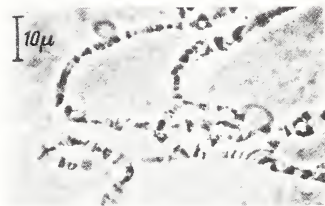
7



8



9

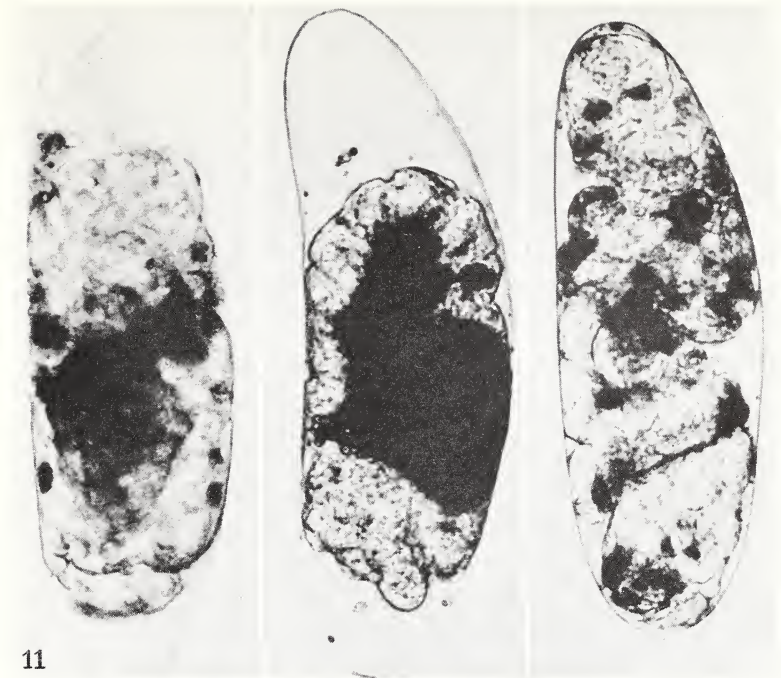


10

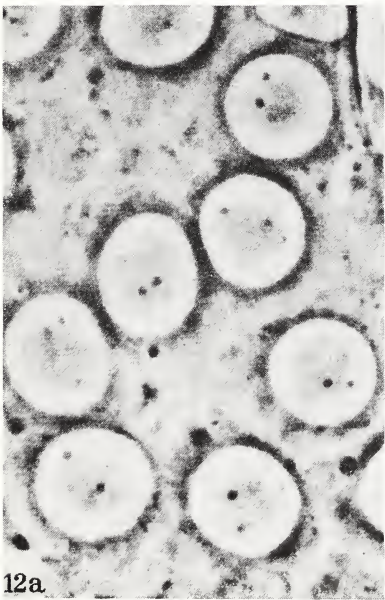
PLATE 52

FIGURE 11.—*Right*: Normally developed larva before hatching. *Middle*: Anucleolate embryo of the same age. *Left*: Anucleolate embryo in the latest stage of development. From Beermann (2).

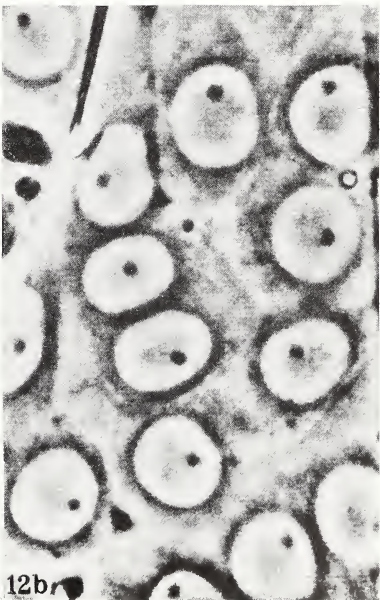
FIGURE 12.—a) Nuclei of anucleolate embryos. b) Nuclei of normal embryos. From Beermann (2).



11



12a



12br

DISCUSSION

Brown: When only the accessory nucleolar organizer is present, can you tell by labeling experiments whether it functions more actively than when normal organizers are present along with the accessory organizer?

Pelling: Unfortunately, one will never get the accessory nucleolus alone in a salivary gland nucleus of an adult larva, because the accessory nucleolus cannot keep the larva alive. Therefore one cannot investigate the RNA synthesis in both types of nucleoli independently. One could compare the synthetic properties of the accessory nucleolus in combination with one, two, three, or four heterozygous nucleolar organizers. We have not done this yet. The accessory nucleolus, it is true, is particularly large if only one heterozygous nucleolar organizing region is present in the same nucleus (Beermann, personal communication).

Busch: I am somewhat confused by Dr. Ritossa's 130 different gene segments that can code for the nucleolus and by some of the data that show there can be 1, 5, 40, and even more nucleoli. One time Dr. Hans Stich was visiting my laboratory and had sections showing what seemed to be hundreds of "micronucleoli" on chromosomes. Now I just would be curious to know how you explain this and whether you see anything similar.

Pelling: It is indeed necessary to distinguish between true nucleoli and the spheres of ribonucleoprotein, which sometimes can be observed at certain RNA synthesizing sites. *Chironomus tentans*, for instance, has two nucleoli, but there is also a small number of other puffs characterized by granular material, early described by Bauer as "Nebennukleolen" (Z. Zellforsch 23: 280-313, 1935). Under certain conditions, particularly in animals, which finish their hibernation period, the number of puffs that show such RNP droplets increases remarkably. Whether the ribonucleoprotein of the nucleolus shows a relationship to the ribonucleoprotein of other gene loci is difficult to decide. Sometimes it looks as if the different gene loci, including the nucleolar organizers, compete with respect to this material. I have just mentioned that the accessory nucleolus becomes larger when it occurs in the nucleus together with only one other heterozygous nucleolar organizer. Also the animals exhibiting so much RNP material in their puffs often seem to have smaller nucleoli.

Pavan: *Rhynchosciara angclae* is a good organism in which to study production of "micronucleoli." I think that in the polytene chromosomes of the salivary gland of this fly every band is potentially capable of producing "micronucleoli." This can be observed in certain stages of larval development, prepupal mainly, in normal larvae, but they are still more evident and in greater number in the case of infected cells (see figs. 1 and 2). I do not think that in this particular instance one should think that *R. angclae* has a diffuse nucleolar organizer region, because this region is clearly located at the base of the X chromosome. Dr. Pelling, in normal larvae of *Chironomus*, are these microbodies produced in different parts of the chromosomes?

Pelling: I must say that, at least in *Chironomus*, the occurrence of these bodies is a very specific phenomenon which is restricted only to puff regions and in most cases only to few of them. It is most improbable that all bands and interbands are active in producing such material.

Pavan: Did you compare your results with the larvae having a deficiency in the nucleolar organizing region with the results obtained by Poulson (Amer Natur 79: 340-363, 1945) and Hadorn (Symp Soc Exp Biol 2: 177-195, 1948) in *Drosophila*? In both instances they noticed that certain lethal mutations would kill the embryos in specific stage of development. My point is this: How would you eliminate the possibility that the deficiency affecting the nucleolar organizer region also includes some neighboring genes vital to the development of the embryos?

Pelling: Because the elimination of the nucleolar organizer has not been achieved by X-ray fragmentation but repeatedly by composition of new and different chromosome complements from the hybrid *Chironomus tentans* \times *Ch. pallidivittatus*, the adjacent genes certainly do not play a preferential role. In some of his crossings Beermann reported special developmental disturbances in correlation with unfavorable chromosome arrangements. It would be nice to compare how similar deficiencies and mutations of the nucleolus and of other genes influence the development of *Drosophila* and *Chironomus*, but real parallel work has not yet been done.

Ritossa: You said that the individuals carrying only a small piece of the nucleolar organizer show a strange behavior. I would like to know something about that.

Pelling: In Beermann's fragmentation experiments, the chromosomes are broken in such a way that individuals carrying the greater part of the organizer have always an additional duplication in the chromosome complement, whereas the smaller portion is necessarily associated with the corresponding deficiency, comprising about 10 to 20 chromosomal bands. Animals with this deficiency and therefore with the smaller nucleolar organizer fragment do not show irregularities during embryonic development, but the larvae cannot hatch from the egg or, if they do hatch, they die soon afterwards. Most probably this inability of further growth is due to the presence of the deficiency. Another explanation certainly cannot be excluded: The number of ribosomal cistrons in the smaller portion of the nucleolar organizer might be too small to maintain viability when the larva is fully autonomous.

Mandel: You said that there is differentiation of nerve and muscle cells. How is it that Nissl's bodies in nerve cells, which are very rich in ribosomes, can form without nucleoli?

Pelling: I do not know whether these embryonic nerve cells contain Nissl's bodies or not, because they have not been investigated histologically. Unfortunately, the nucleolus-less stock is no longer available, so that we cannot check this.

Schultz: I should like to put this discussion into a more general context, considering the nucleolar organizer as an extreme example of a complex locus. What we do know about most of the genes in multicellular organisms that have been analyzed in detail genetically is that they have turned out to be complex loci with many cistrons. Take such cases as the white locus or others in *Drosophila* studied by such workers as Lewis, Green, Chovnick, etc. These may have as many as 5 separable subunits. From this point of view, the nucleolar organizer is a massive accumulation of representatives of a locus to do a special function, essential in almost all cells. The beauty of the work Dr. Ritossa has presented, in particular that with respect to the *bobbed* locus, is that here the genetic data have long indicated the *bobbed* locus to be complex, but genetic techniques could not be applied fruitfully simply because the genetic markers were not adequate. From their work arises the enormously attractive opportunity to check the test of activity genetic mutants give us, by the structural test of DNA sequence afforded by hybridization experiments at the molecular level.

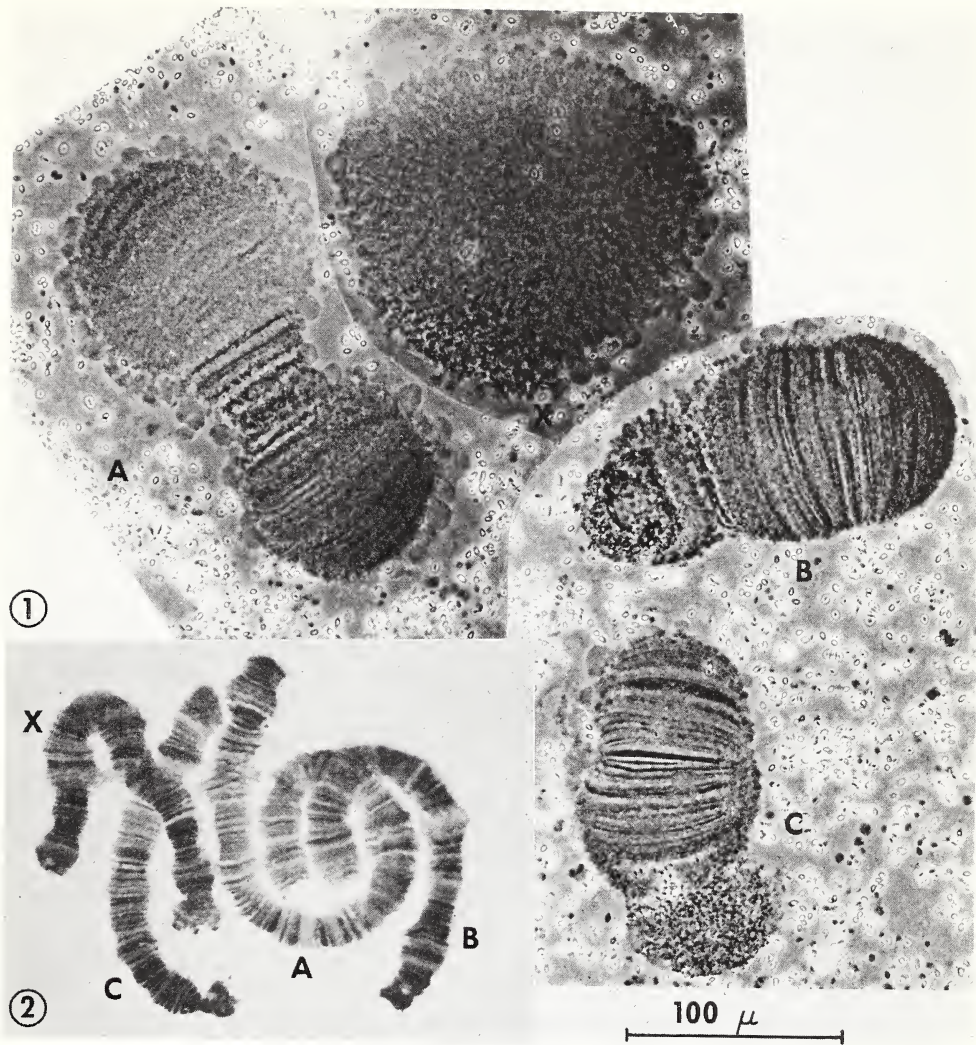
I have a point to make about Dr. Pelling's discussion of nucleolar function, *i.e.*, to mention the information available from Dr. von Borstel's experiments on the development of eggs without a nucleolus in *Drosophila* [Nature (London) 181: 1597-1598, 1958]. I had hoped he would say something about his own investigation on the nucleolus-less state in *Drosophila*. He was able to show that development stops at a very early stage, before the blastoderm is formed, which is consonant from all that we know from the *Xenopus* and the *Chironomus* studies.

PLATE 53

FIGURE 1.—The polytene chromosomes (A, B, C, and X) from a salivary gland cell infected by a protozoan, probably a microsporidian. Infected cells, as well as their chromosomes are greatly enlarged and many bands well puffed. In many cases, like the X chromosome in this figure, the banding pattern is practically lost and the chromosome is entirely puffed. After a certain stage of the infection it is common to observe many of these microbodies, which we call nucleoloides, on the surface of practically all chromosomes.

FIGURE 2.—The polytene chromosomes of a normal cell of the salivary gland of *Rhynchosciara angelae*.

The magnification is the same for both figures.



Problems in the Developmental Cytogenetics of Nucleoli in *Xenopus*^{1, 2}

H. J. BARR,³ *Department of Zoology, University of Wisconsin, Madison, Wisconsin*

SUMMARY

A brief outline is given of the cytological and embryological studies that have been made of the Oxford nucleolar factor in *Xenopus laevis*. The previous demonstration by Esper and Barr of nucleoli in "anucleolate" embryos, *i.e.*, embryos homozygous for this factor, is discussed.

The original observations on this subject by Esper and Barr are extended to include the finding of nucleoli in cells of tissues derived from all three germ layers of the homozygous mutant embryos.—*Nat Cancer Inst Monogr* 23: 411–424, 1966.

MY ASSIGNED TASK in this paper is to outline the cytological and embryological studies that have been carried out on the Oxford nucleolar factor in *Xenopus* (1) and to discuss the implications of these studies for our eventual understanding of this mutant system which has recently yielded biochemical information of great interest (2, 3).

THE PATTERN OF INHERITANCE OF THE OXFORD NUCLEOLAR FACTOR

The cells of wild-type *Xenopus* may contain during interphase either two nucleoli or one. It is assumed that there are in such cells two nucleolar organizing regions (NORs), one associated with each haploid set of chromosomes. Where two nucleoli are found, each is assumed to be associated with one such NOR, and where only one nucleolus is found it is assumed either that its assembly is due to the cooperation of the two NORs or that it results from the fusion at some stage in the cell cycle of two nucleoli, each associated with one NOR.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This work was supported by Public Health Service research grant GM-13549-01.

³ I wish to thank Dr. Walter Plaut for the opportunity of working in his laboratory, and Mr. Donald E. Chandler for the photomicrography.

The Oxford nucleolar factor was first discovered in a female *Xenopus* which produced some eggs that, when fertilized by wild-type sperm, developed into diploid larvae whose interphase cells contained one, but never more than one, nucleolus. Such larvae appeared otherwise wild-type, developed normally, metamorphosed, and grew into fertile adult males and females, morphologically indistinguishable from wild-type adults.

When crossed in either direction with wild-type animals, such adults produced progeny half of which were wild type and half of which showed the same nucleolar effect as their mutant parents, *viz.*, interphase nuclei containing one nucleolus only. It is possible to cross two such mutant individuals; such crosses yield 25% wild-type individuals, 50% mutant individuals like their parents, and 25% offspring which die as larvae. Members of this last class, discussed below, contain during their early stages no proper nucleoli and were therefore very reasonably termed "anucleolate" by Wallace, to whom we are indebted for having worked out in considerable detail their cytology and embryology (4-8).

A simple and reasonable explanation of these observations was that the original female among whose progeny this factor was found was germinally mosaic for a factor which, when present heterozygously, prevents the appearance of a nucleolus associated with one of the two NORs of the cell. When present homozygously, such a factor would lead to the absence of proper nucleoli from the cell. It should be pointed out that this explanation makes no assumptions about the chromosomal location of the factor involved. A more explicit form of this explanation is that the factor is either a physical deletion of all or part of the NOR, or a mutation of a controlling element which governs the functioning of the NOR. Such a controlling element might be adjacent to, or not at all linked to, the NOR.

The only direct approach to the cytological mapping of this factor was made by Kahn (9) whose karyological studies demonstrated a secondary constriction on a certain pair of chromosomes in wild-type individuals. The presence of the mutant factor was correlated with the absence of this secondary constriction. Since NORs are frequently, but not universally, associated with secondary constrictions, it is very likely that this correlation is not fortuitous. The absence of a secondary constriction may be a reflection of the physiological state of a chromosome region, or it may be due to a deletion for the region that, when present, forms the constriction. There is certainly no evidence in Kahn's interesting findings that would *necessarily* lead to a conclusion that the Oxford factor is a deletion for the NOR. The chromosomal locations of NORs in *Xenopus* remain unknown.

THE HETEROZYGOTES

Fischberg and Wallace (4) reported of cells heterozygous for the Oxford factor that "... their single nucleolus is larger than either

nucleolus of the normal two-nucleolated cells." This subject was studied further by Dr. Hildegard Esper and myself (10), who examined the three classes of nucleolus then known in *Xenopus*: (a) the paired nucleoli found in some wild-type cells, (b) the single nucleolus found in other wild-type cells, and (c) the single nucleolus found in cells heterozygous for the Oxford factor. Our data, based on replicate measurements of each of 200 nucleoli in chondrocytes from stage 43 larvae (11), showed that the single nucleus in heterozygous cells (class c) achieves the same volume as the nucleolus in wild-type cells having only one nucleolus (class b). This result is discussed in our 1963 paper in some detail with reference to what has been called "nucleolar competition" (10).

An observation made in 1954 by McLeish (12) (of which, unfortunately, Dr. Esper and I were unaware when we wrote our 1963 paper) showed that as a result of abnormalities produced with 8-ethoxycaffeine or maleic hydrazide in *Vicia faba*, some cells containing two nuclei—a major nucleus and a micronucleus—were formed. In some, the major nucleus contained only one NOR, and the nucleolus formed in association with this single NOR (analogous to our class c nucleoli) fell into the same size range as the single, "fused" nucleolus found in a proportion of normal cells which contained two NORs (analogous to our class b nucleoli). The fact that this "compensatory" increase in the size of a remaining nucleolus in the absence of a functional homologue occurs in two such phylogenetically disparate organisms as *Vicia* and *Xenopus* suggests that it may be a phenomenon of some generality and not an isolated curiosity. Granting this, we may then ask whether this "compensatory" phenomenon is peculiar to NORs or is characteristic of chromosomal regions in general. Does the study of the various classes of nucleolus in *Xenopus* perhaps offer a model system for the study of dosage compensation, the single-active-X hypothesis, or even dominance itself? I have no answer to this question, but the fact that it can be seriously raised suggests that further cytogenetical analysis of the Oxford system may prove fruitful.

At this point I should like to note an assumption frequently made but not previously explicated: that class (c) nucleoli are associated with one functional NOR, whereas class (b) nucleoli are associated with two. How do we know that the single, "fused" nucleolus (class b) found in wild-type cells is in fact *either* the result of fusion of two nucleoli, each associated with one NOR, *or* the result of cooperation between the two NORs of the wild-type cell? May it not be that in wild-type cells having a single "fused" nucleolus only one of the two NORs present is functional? On this view, the presence of only one nucleolus in some wild-type cells would be due to the inactivation in those cells of one NOR. The remaining nucleolus would become larger than either of the paired nucleoli found in some wild-type cells, not because of the operation of two NORs in its formation, but because it was exhibiting "compensation," just as the single nucleolus in a heterozygous cell is thought to do. The analogy here with the single-active-X hypothesis is obvious.

In *Triturus*, Amenta (13) has directly observed both nucleolar fusion and separation, but no such study has been made with *Xenopus*. Wallace (14) in fact excludes the possibility that nucleoli fuse during interphase in *Xenopus* as they have been shown to do in *Vicia* by Woodard (15). Evidence for the physical association of regions of several different chromosomes with one nucleolus has been presented for human cells by Ferguson-Smith and Handmaker (16), and Ohno *et al.* (17) have suggested that “. . . not all nucleolus organizing regions are functional at the same time . . .” and that, with respect to nucleolar organizing activity, “. . . there can be one active and one inactive member of a single chromosome pair.”

THE HOMOZYGOTES

I wish to turn now to the cytological and developmental studies that have been carried out on embryos homozygous for the Oxford factor. Wallace showed that these embryos develop with normal external morphology until hatching, after which time their development is retarded and abnormal; within a few days, all such larvae die. Histological studies reveal excessive pyknosis in various ectodermal and endodermal derivatives before hatching and some aspects of morphogenesis, *e.g.*, of the pharyngeal epithelium, are abnormal. Such embryos were correctly reported by Wallace to contain no normal nucleoli at the stages he studied, but rather a variable number of small particles termed “blobs,” a point confirmed by Dr. Esper and myself [*see* figs. 1 and 2 in (6) and fig. 1 in (18)]. These blobs were shown by Wallace to contain RNA, an arginine-rich protein and alkaline phosphatase. He further showed that cytoplasmic RNA, as stained with the methyl green-pyronine technique and estimated visually, fails to show the same increase during development in homozygous mutant embryos as is shown by wild-type and heterozygous embryos.

Wallace initiated an extensive series of embryological transplants designed to show whether the homozygous mutant condition is a cell lethal. Homozygous mutant embryos (ONU) were combined with wild-type or heterozygous embryos (+NU) in parabiosis, and tails were interchanged between +NU and ONU tailbud embryos. All anucleolate tissues survived for at least twice, and some as long as 4 times, their usual lifespan. Since some intermingling of +NU and ONU cells occurs, the possibility of overgrowth of ONU cells by +NU ones cannot be ruled out. Furthermore, Wallace presents evidence suggestive of immunological intolerance in his grafts: +NU leukocytes are seen destroying some ONU tissues. For these reasons, these transplantation experiments cannot directly test for a cell lethal in the sense that term is used by *Drosophila* workers (19); Wallace refers to the ONU condition as a subvital. Given the failure of the circulation, the death and lysis of some cells, and the possibility of attack by bacteria, it is not at all surprising that these *embryos* eventually

die; there is, however, no evidence that their *cells* might not continue to live *in vitro*.

With the foregoing data in mind, as well as Wallace's pioneering chemical studies, which showed a marked deficiency of total RNA in ONU embryos, Dr. Esper and I studied quantitatively, by cytophotometric measurement of Azure B dye binding, the amount of RNA in the cytoplasm of various cell types during development (18). While our paper was in press, the important contribution by Brown and Gurdon appeared (2). These two approaches, the biochemical and cytological, complement each other in that, while cytochemistry cannot as yet distinguish among classes of RNA, biochemical procedures, applied to whole embryos, cannot resolve differences among cell types or correlate those differences with morphological data. Since the bulk of cellular RNA is ribosomal, we were very likely to be measuring that fraction with our techniques, but we pointed out dangers in this assumption (20, 21).

Very briefly, our study yielded two surprising results. *First*, the difference in cytoplasmic RNA concentration between +NU and ONU embryos varied from tissue to tissue. In pharyngeal epithelium the concentration of RNA per unit volume of cytoplasm increases considerably in the +NU embryos during the developmental stages studied, while in the ONU embryos this figure remains at its initial level of about half the +NU value. In the spinal cord there is a slight rise during development in the +NU embryos, but the ONU value shows no increase and remains about 20% below the +NU level. In pronephric tubules, however, there is no difference in the concentration of RNA per unit volume of cytoplasm between +NU and ONU embryos at the stages studied. And in this tissue our data actually show an *increase* during development of total cytoplasmic RNA per cell in the homozygous mutants.⁴

This finding is less disturbing in the light of our *second*, even more surprising, result: At the later stages studied, ONU, "anucleolate" embryos contain nucleoli. These particles are indistinguishable from +NU nucleoli on the basis of their staining reactions with the Kurnick-Ris stain or Azure B. In the pronephros of ONU embryos, the largest nucleoli are statistically indistinguishable in size from the nucleoli of +NU embryos of the same age. We reported nucleoli in other tissues as well; in the pharyngeal epithelium and spinal cord the largest ONU nucleoli were, however, somewhat smaller than their +NU counterparts.

Since our original paper on the homozygotes, I have extended these observations and can now report, on the basis of a total of three separate matings involving six different heterozygous parents, that nucleoli are formed in the ONU embryos in all cases. Among the tissues that show nucleoli of normal or nearly normal size in ONU embryos at a time when their +NU control sibs are at stage 46 are: nerve, epidermis, mesen-

⁴ I am indebted to Dr. David R. Sonneborn for pointing out the possible relevance to these findings of the observation by Waddington (22) and Lasansky and De Robertis (23) of an increase in cytoplasmic RNA granules in cells destined soon to die.

chyme, muscle, cartilage, pronephros, gut epithelium, and liver. Figures 1 through 4 illustrate this phenomenon for ONU muscle, liver, pronephros, and cartilage, respectively.⁵

I have now seen ONU cells containing two nucleoli of equal size as well as those containing a single, large nucleolus (fig. 4).

The implications of finding nucleoli in ONU embryos have been discussed at length against the background of nucleolar cytogenetics by Dr. Esper and myself. Here I wish to point out only that the new observation of two nucleoli in some ONU cells makes it possible that in such cells both of the usual NORs have been activated.

Do the nucleoli of ONU cells result from the fusion of the blobs found at earlier stages in these cells? If so, what changes occur within the environment of these nuclei to cause this fusion? Are these changes the result of differential gene activity or are they the result of less specific environmental factors, *e.g.*, the accumulation within these nuclei of magnesium ions? And what is it about being fused into one or two particles that is so important for nucleolar function?

Alternatively, if the nucleoli found in ONU cells are *not* formed by the fusion of the blobs found at earlier stages, what is the genetical or physiological explanation of their delayed appearance? Are they associated with the usual NORs? If so, is the Oxford factor a mutation in a controlling element responsible for the timing of NOR function during development, a subject recently studied by Mintz (25)? Or are the nucleoli of ONU cells formed by the activity of what have been called accessory NORs (26-29)?

The fact that magnesium deficiency can produce a phenocopy of at least the external morphology of the homozygous condition suggests that water and ion balance may be involved in the mechanism of action of the Oxford factor (2). Magnesium is, after all, requisite for the structural integrity of ribosomes and possibly for nucleoli as well. Numerous developmental disturbances in amphibians are associated with abnormalities of water balance (30, 31), and at least one hereditary abnormality in the axolotl can be "cured" by adding certain inorganic ions to the medium (32). Certainly such ions are not without specific developmental effects in amphibians (33, 34). In fact, general theories of development based on ionic phenomena have been offered by Willmer and Kroeger (35, 36).

If the Oxford factor results in raising the threshold value of Mg^{+2} requisite for assembly of a nucleolus at the NOR of the haploid set carrying the mutant factor, then the appearance of nucleoli in ONU embryos could be explained by an increasing level of Mg^{+2} within the nuclei during

⁵ Stenram's (24) observation of a marked increase in nucleolar size in the cells of rats fed on diets deficient in certain essential amino acids should be borne in mind. Since Wallace (6) has shown that the digestion of intracellular yolk platelets is retarded in ONU embryos, possibly the nucleoli of ONU embryos are produced in response to a deficiency of amino acids.

It should be noted that Wallace (7) observed the blobs of ONU embryos parabiosed for 10 days to +NU ones to be larger than those of unoperated ONU embryos of the same age; these, he found, "... become even larger ..." in the ONU cells of older parabiotic pairs. He may thus have been observing the formation of nucleoli in ONU cells.

development. Alterations in the permeability of the nuclear envelope during development are known to occur (37).

These possibilities are mentioned because they suggest experiments and because they provide an alternative to the general view that developmental mechanisms are coextensive with selective gene activation. On this alternative view the normal development of the embryo depends in part on a specific geometrical arrangement of cell constituents (the nucleolus) and the presence or absence of this arrangement may depend on factors within the cell (ionic) that are not themselves controlled by the operation of specific cistrons.⁶

RESUMEN

Se presenta una breve reseña de los estudios citológicos y embriológicos que se han realizado sobre el factor nucleolar Oxford en *Xenopus laevis*.

Se discute la demostración previa de Esper y Barr de los nucleolos en los embriones "anucleolados" esto es, los embriones homocigóticos para este factor.

Se extendieron las observaciones originales de Esper y Barr sobre este tema de modo de incluir el hallazgo de nucleolos en células de tejidos derivadas de las tres capas germinales de los embriones mutantes homocigóticos.

REFERENCES

- (1) ELSDALE, T. R., FISCHBERG, M., and SMITH, S.: A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp Cell Res* 14: 642-643, 1958.
- (2) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (3) GURDON, J. B., and BROWN, D. D.: Cytoplasmic regulation of RNA synthesis and nucleolus formation in developing embryos of *Xenopus laevis*. *J Molec Biol* 12: 27-35, 1965.
- (4) FISCHBERG, M., and WALLACE, H.: A mutation which reduces nucleolar number in *Xenopus laevis*. In *The Cell Nucleus* (Mitchell, J. S., ed.). New York, Academic Press Inc., 1960.
- (5) WALLACE, H.: The development of anucleolate embryos of *Xenopus laevis*. *J Embryol Exp Morph* 8: 405-413, 1960.
- (6) ———: Cytological and biochemical studies of anucleolate *Xenopus* larvae. *Quart J Micr Sci* 103: 25-35, 1962.
- (7) ———: Prolonged life of anucleolate *Xenopus* tadpoles in parabiosis. *J Embryol Exp Morph* 10: 212-223, 1962.
- (8) ———: Tail grafts involving anucleolate *Xenopus* embryos. *Develop Biol* 5: 252-263, 1962.
- (9) KAHN, J.: The nucleolar organizer in the mitotic chromosome complement of *Xenopus laevis*. *Quart J Micr Sci* 103: 407-409, 1962.
- (10) BARR, H. J., and ESPER, H.: Nucleolar size in cells of *Xenopus laevis* in relation to nucleolar competition. *Exp Cell Res* 31: 211-214, 1963.

⁶ An alternative to the idea that developmental mechanisms and selective gene activation are coextensive, namely, a model for the generation of complex patterns by the operation of simple recursive rules, has recently been developed with vigor and cogency by Braverman and Schrandt (38).

- (11) NIEUWKOOP, P. D., and FABER, J.: Normal Table of *Xenopus laevis*. Amsterdam, North-Holland Publ Co., 1956.
- (12) McLEISH, J.: The consequences of localised chromosome breakage. *Heredity* 8: 385-407, 1954.
- (13) AMENTA, P. S.: Fusion of nucleoli in cells cultured from the heart of *Triturus viridescens*. *Anat Rec* 139: 155-166, 1961.
- (14) WALLACE, H.: Nucleolar growth and fusion during cellular differentiation. *J Morph* 112: 261-278, 1963.
- (15) SWIFT, H.: Studies on nucleolar function. In A Symposium on Molecular Biology (Zirkle, R. E., ed.). Chicago, Univ Chicago Press, 1958, pp 266-303.
- (16) FERGUSON-SMITH, M. A., and HANDMAKER, S. D.: The association of satellited chromosomes with specific chromosomal regions in cultured human somatic cells. *Ann Hum Genet* 27: 143-156, 1963.
- (17) OHNO, S., KAPLAN, W. D., TRUJILLO, J. M., and KINOSITA, R.: Nucleolus-organisers in the causation of chromosomal abnormalities in man. *Lancet* 2: 123-125, 1961.
- (18) ESPER, H., and BARR, H. J.: A study of the developmental cytology of a mutation affecting nucleoli in *Xenopus* embryos. *Develop Biol* 10: 105-121, 1964.
- (19) HADORN, E.: Developmental Genetics and Lethal Factors. New York, John Wiley & Sons, 1961.
- (20) DEANE, H. W., and PORTER, K.: A comparative study of cytoplasmic basophilia and the population density of ribosomes in the secretory cells of the mouse seminal vesicle. *Z Zellforsch* 52: 697-711, 1960.
- (21) MOULÉ, Y.: Endoplasmic reticulum and microsomes of rat liver. In Cellular Membranes in Development (Locke, M., ed.). New York, Academic Press Inc., 1964.
- (22) WADDINGTON, C. H.: New Patterns in Genetics and Development. New York, Columbia Univ Press, 1962.
- (23) LASANSKY, A., and DE ROBERTIS, E.: Submicroscopic analysis of the genetic distrophy of visual cells in C3H mice. *J Biophy Biochem Cytol* 7: 679-684, 1960.
- (24) STENRAM, U.: Effects of diet on the nucleolus. *Exp Cell Res, Suppl* 9: 176-181, 1963.
- (25) MINTZ, B.: Synthetic processes and early development in the mammalian egg. *J Exp Zool* 157: 85-100, 1964.
- (26) CHOUINARD, L. A.: Sites of formation of the extra nucleoli during early oocyte growth in the freshwater teleost *Salvelinus fontinalis* Mitchell. *Canad J Zool* 41: 997-1010, 1963.
- (27) GABRUSEWRCZ-GARCIA, N.: Studies of nuclear RNA in the salivary gland *Sciara coprophila* (Abstract). *J Cell Biol* 27: 31A, 1965.
- (28) KOPAC, M. J., and MATEYKO, G. M.: Nucleolar chromosomes: structures, interactions and perspectives. *Advances Cancer Res* 8: 121-190, 1964.
- (29) VINCENT, W. S.: Structure and chemistry of nucleoli. *Int Rev Cytol* 4: 269-298, 1955.
- (30) DEYRUP, I. J.: Water balance and kidney. In Physiology of the Amphibia (Moore, J. A., ed.). New York, Academic Press Inc., 1964.
- (31) TUFT, P.: Distribution of water in the embryo. *Nature (London)* 191: 1072-1073, 1961.
- (32) JUSTUS, J. T., and HUMPHREY, R. R.: The effects of sodium, potassium, and calcium ions on certain expressions of the semilethal gene *V* in the Mexican axolotl, *Ambystoma* (= *Siredon*) *mexicanum*. *Develop Biol* 9: 255-268, 1964.
- (33) BÄCKSTRÖM, S.: A link between neural induction and vegetalization. The effect of magnesium on *Xenopus* embryos. *Acta Embryol Morphol Exp (Palermo)* 5: 295-303, 1962.

- (34) BARTH, L. G., and BARTH, L. J.: The relation between intensity of inductor and type of cellular differentiation of *Rana pipiens* presumptive epidermis. Biol Bull 124: 125-140, 1963.
- (35) WILLMER, E. N.: Cytology and Evolution. New York and London, Academic Press Inc., 1960.
- (36) KROEGER, H.: Experiments on the extranuclear control of gene activity in dipteran polytene chromosomes. J Cell Comp Physiol 62, Suppl 1: 45-59, 1963.
- (37) ITO, S., and LOEWENSTEIN, W. R.: Permeability of a nuclear membrane: changes during normal development and changes induced by growth hormone. Science 150: 909-910, 1965.
- (38) BRAVERMAN, M. H., and SCHRANDT: Colony development of a polymorphic hydroid as a problem in pattern formation. In The Cnideria and Their Evolution (Rees, W. J., ed.). New York and London, Academic Press Inc., 1966.

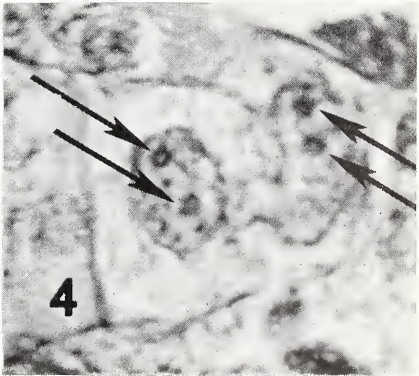
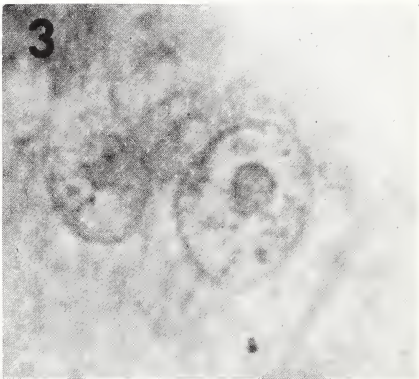
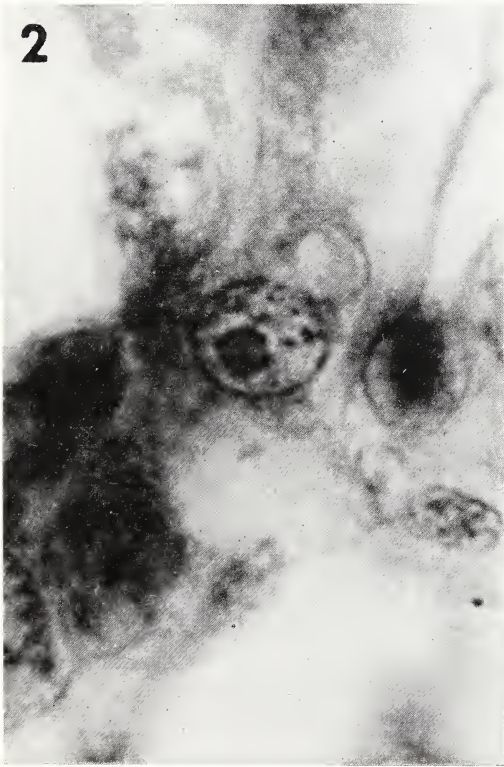
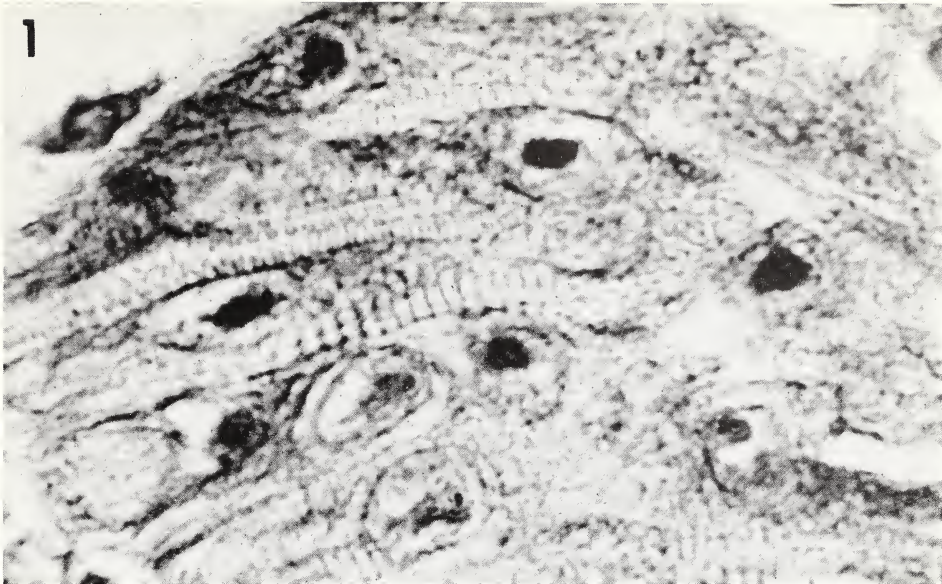
PLATE 54

FIGURE 1.—Section from a ONU embryo showing nucleoli in muscle. Azure B.
× 810

FIGURE 2.—Section from a ONU embryo showing a nucleolus within the nucleus
of a liver cell. Kurnick-Ris. × 810

FIGURE 3.—Section from a ONU embryo showing a nucleolus within the nucleus
of a pronephric cell. Kurnick-Ris. × 810.

FIGURE 4.—Section from a ONU embryo showing two chondrocytes, the nucleus
of each of which contains two nucleoli (*arrows*). Azure B. × 810



DISCUSSION

Birnstiel: Our results in *Xenopus* admittedly cannot show that all of the ribosomal cistrons have been deleted. At present the hybridization techniques are not sensitive enough to detect a solitary residual cistron in higher organisms. So the mutant may, in fact, contain a very few ribosomal cistrons. We can show that there is a linear reduction of the ribosomal cistrons with the dosage of the mutation and whether the intercept in the homozygous mutant goes through zero or 5% of the wild-type value does not affect our arguments.

Brown: If 5% of the DNA complementary to ribosomal RNA (rRNA) is left, the anucleolate embryos still make no detectable rRNA whatsoever at any time during their lifetime, even when these nuclear bodies are present. Can you describe some cytochemical features of these nuclear bodies? Are they ribonuclease-sensitive?

Barr: It is possible that increase in total RNA in the cytoplasm of the pronephros of these anucleolates may not be due to ribosomes. Dr. Wood showed that the sections from which he had extracted transfer RNA (tRNA) stained far more lightly than did sections which did not have the tRNA extracted. So we may be dealing in differences from tissue to tissue of RNAs other than rRNA. But it is still interesting that the situation is different in different tissues. It may simply be that different cells have different amounts of rRNA to begin with. There is some work, mentioned by Dr. Waddington in one of his books, where cells in insect eyes that are destined to die show a marked increase in Palade granules in the cytoplasm. Lasansky and De Robertis (23), have shown a similar thing in genetic dystrophy in retinal cells in the mouse. Cells which are to die soon for some reason or other collect ribosome-like particles in the cytoplasm. The RNA staining is removed with ribonuclease, but we have made no photometric measurements on them.

Lettré: I should like to cite a paper of W. H. Lewis (Lewis, Anat Rec 97: 433-446, 1947) which perhaps may be related to the observation of blobs. When through an abnormal mitosis a great number of micronuclei originate, many of them contain dark blobs. According to our own observations (Lettré and Siebs, unpublished observations) more blobs than the number of nucleolar organizers present may be found. This means that in the normal nucleus some material which obviously does not ordinarily become visible becomes apparent when isolated chromosomes form micronuclei. This could be material which in the presence of a nucleolus would not accumulate.

Barr: Dr. Wallace pointed out in his original paper that these particles resemble what happens in some of the disturbed cells that Dr. McClintock studied in maize where you get small blobs all over.

Pavan: I have three questions. First, in one of your previous papers [table 1, Barr and Esper, Exp Cell Res 31: 211-214, 1963 (10)] I noticed that you measured the volume of the nucleoli in the wild type having two nucleoli, in the wild type having a fused nucleolus, and in the heterozygote with just one nucleolus. The results indicate that when you measure the wild type with two nucleoli, the total volume is smaller than the fused one in the wild type or the single one in the heterozygote. My question is this: Do you think that you have more gene activity when the nucleoli in the wild type are together than when they are separate? The second question concerns the nucleolar-like globules that you find in the anucleolate *Xenopus*. I would like to point out to the Nomenclature Committee that it is very necessary to have a name for all the structures inside of the nucleus which look like nucleoli but are not nucleoli. The third question: I would like to know if Dr. Barr can tell us something about the relation between the development of the embryo in a case

where the mutant has a deficiency which is not in the nucleolar organizing region but in which a nucleolus does not form, and the development of the embryo where the mutant has a deletion in the nucleolar organizing region.

Barr: Well, first, about the volume, Dr. Swift, do you want to comment on that?

Swift: There are several reports in the literature (*e.g.*, Bhatia, *Ann Bot* 2: 335, 1938; Parthasarathy, *Cytologia* 9: 397, 1938, and our own measurements) that when two nucleoli fuse together their volume is characteristically larger than the combined volume of two unfused nucleoli. This is apparently also true when one organizer is lost. If material entered and left the nucleolus as a function of its surface area rather than its volume, this is the relationship one would expect. Thus we might postulate that the nucleolar surface is important in collecting material made elsewhere, such as ribosomal proteins from the nucleoplasm, or in the loss of newly assembled precursors of the ribosomes.

Barr: Dr. Pavan's second question concerned nomenclature. I would like to disagree with you head-on, Dr. Pavan, and address a comment to you, and maybe to the Nomenclature Committee. I am not sure that we advance our understanding of problems by giving nice, in the literal sense of "nice," anatomical names to things. There is a famous quote by the Harvard logician Quine that the less a science is developed, the more it tends to rest on uncritical assumptions of mutual understanding. It is a very true quote, but it does not follow that the way to develop a science is to get a lot of definitions. I think that as we work out the problems, as we find out more about the formation of nucleoli, our terminology will develop as we know what is going on. We may simply hamper ourselves by creating lots of terminology at this point. I think that it is better to be concerned with the details of what is going on rather than with whether we should call these things mock nucleoli, blobs, or what. I want to answer, at this point, your third question which I think is very important. Dr. McClintock's original, classic paper on nucleolar organizers was done with maize (*Z Zellforsch Mikroskop Anat* 21: 294-328, 1934). In this material the meiotic prophase chromosomes are long enough so that one can readily observe them and can identify regions along them. The region of the nucleolar organizer, as defined as the place where one sees the nucleolus, was mapped. Now, among many other things, Dr. McClintock found a deficiency, a strict physical deletion, that is not located anywhere near the nucleolar organizer region of the maize karyotype. However, in the presence of this deficiency, nucleoli do not form at the usual nucleolar organizing region and, further, you do not see the secondary constrictions at the nucleolar organizing regions. The secondary constriction is the result of a nucleolus' having been formed at a certain region. Now, what I would like to make clear here is that Dr. Birnstiel *et al.* have unquestionably proved that there is a deletion. Their 2, 1, 0 data prove that beyond question. But, they haven't shown where in the genome, where in the karyotype of *Xenopus*, it sits. It is logically possible that this deletion is not at the region which in *Xenopus* is the nucleolar organizing region. In other words, it might be like the deletion described by Dr. McClintock. It seems to me a rather nice way to put a cell together, to have the rRNA cistrons right at the region which is the nucleolar organizer; but we haven't yet proved that. There is a deletion. We have no data to prove where it is. It is very likely at the site of the organizer, but we don't yet know for sure.

Kasten: I would like to ask a question which relates to some of the quantitative data that were presented. I did not notice what the percentage differences were between the mean amounts of RNA in the two cell types, the anucleolate and the other, nor did I notice any statistical analysis. Judging from the table [table 2, Esper and Barr, *Devel Biol* 10: 105-121, 1964(18)], it looked as if the standard errors of the mean for most of these data were in the range of about 6 to 7%. I personally admire your courage in making cytoplasmic basophilic measurements, first because of the great problems involved in getting reproducible results where the cytoplasmic staining is irregular, and second, because of using the plug method to

obtain these data. Did you carry out replicate measurements in several places in the cytoplasm of each cell? Did you make any statistical analyses to demonstrate significant differences between the groups?

Barr: I thank you for admiring my courage. The answer is yes, there is statistical analysis in the paper. In one instance the difference in amounts of RNA is on the order of 300%. I happen to share your worries about measurements of cytoplasmic basophilia. However, when the epithelium gives, on our particular arbitrary scale, something on the order of 0.4 in the ONU and 0.8 to about 1.3 in the +NU, I think that we demonstrate clear-cut differences. I think the smallest difference we have anywhere is 20%, and if it were any closer than that I would be very unhappy; but when we deal with a 300% difference I think we are safe, even with these methods.

Autoradiographic Studies of RNA and Protein Synthesis in Anucleolate *Xenopus* Embryos¹

H. WALLACE, *Department of Biology, Amherst College, Amherst, Massachusetts*

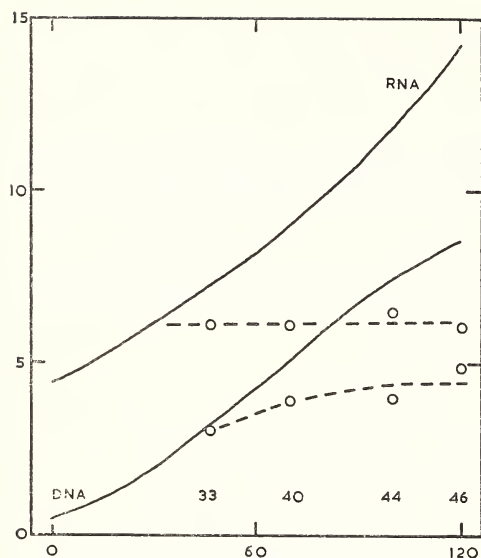
SUMMARY

The incorporation of ³H-uridine and methyl-¹⁴C-methionine into somitic muscle was studied in axial fragments of anucleolate and control *Xenopus* embryos. ³H-uridine incorporation is restricted to the nuclear RNA during incubations up to 3 hours. The nuclei of wild types and heterozygotes show a partition of incorporation (grain counts) of one third into nucleoli and two thirds into chromatin. The nuclei of anucleo-

late mutants incorporate, without any evident localization, an amount equivalent to that of the wild-type chromatin. Methyl-¹⁴C-methionine is incorporated both into protein and into RNA. Pretreatment with puromycin reduces this incorporation (presumably of the protein component), which results in uniform labeling apart from higher grain counts over nucleoli.—Nat Cancer Inst Monogr 23: 425–430, 1966.

SEVERAL YEARS have elapsed since the original attempts to define the lethal syndrome, and to estimate the nucleic acid content, of *Xenopus* mutants which lack nucleoli (1, 2). More refined data of the amount, base composition, and sedimentation characteristics of *Xenopus* nucleic acids are now available from the studies of Bristow and Deuchar (3, 4), Brown and Littna (5, 6), and Brown and Gurdon (7). The total content of both RNA and DNA of the normal embryo increases steadily from soon after fertilization until the feeding larval stage, beyond which anucleolate mutants do not survive. Text-figure 1 shows the net synthesis of RNA and DNA by normal *Xenopus* embryos, taken from the data of Bristow and Deuchar (3), together with my own results for anucleolate mutants. My results, partly recalculated from older data (2), show a considerable variability according to the size of eggs obtained from different parents. This text-figure illustrates the basic fact that anucleolate embryos do synthesize some RNA, but that their synthesis of RNA is suppressed more stringently and earlier in development than is their synthesis of DNA.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

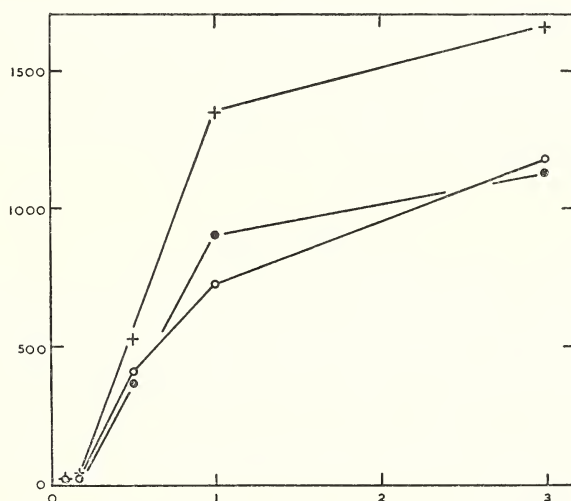


TEXT-FIGURE 1.—Nucleic acid content of *Xenopus* embryos. Ordinate: micrograms nucleic acid per embryo; abscissa: age in hours, with inset stage numbers of Nieuwkoop and Faber (8). Solid lines taken from data of Bristow and Deuchar (8); broken lines are the equivalent values for anucleolate mutants.

Further analysis of RNA metabolism has shown that *Xenopus* embryos do not synthesize ribosomal RNA before gastrulation (5), and that the principal defect of anucleolate mutants is their inability to synthesize ribosomal RNA (7). The preceding article provides evidence that this mutation is a deletion of the nucleolar organizer, which contains the genetic information for ribosomal RNA and is thus essential for its synthesis (see also 9, 10). We cannot, of course, decipher the exact relationship between the ribosomal cistrons and the nucleolar organizer. This mutation, apparently a deletion of all the ribosomal cistrons, may involve only a vital part of the nucleolar organizer, or may comprise the entire nucleolar organizer and perhaps small neighboring segments of the chromosome. The mutation does not directly affect the synthesis of soluble RNA, however, which thus forms the major type of RNA to be accumulated by anucleolate embryos (7). This situation should provide us with an opportunity to determine if the sites of synthesis of soluble RNA are localized or dispersed throughout the chromatin of anucleolate mutants, and perhaps to determine if such soluble RNA cistrons or the soluble RNA itself is normally collected to the site of the nucleolus.

For this purpose, autoradiographs were made of the incorporation of ^3H -uridine by anucleolate and control embryos, at stage 33 of Nieuwkoop and Faber (8). The axial regions of these embryos were excised, divided into several fragments, and incubated for periods between 5 and 180 minutes in a saline solution containing 0.1% albumin and 45 $\mu\text{C}/\text{ml}$ ^3H -uridine. The fragments were then fixed in cold trichloroacetic acid, sectioned at 2.5 μ , and covered with Kodak AR10 stripping film for exposures lasting between 3 and 80 days. Grains were counted over 10 nuclei in longitudinal sections of the somitic muscles. All fragments showed similar patterns of

incorporation: Cytoplasmic uptake and background radioactivity were negligible in comparison to nuclear incorporation. The nuclear incorporation showed a lag period of about 10 minutes, followed by rapid incorporation during the first hour of incubation and a slower incorporation during the following 2 hours (text-fig. 2). Approximately one third of the nuclear incorporation of control tissues was into the nucleolus, the remaining two thirds being localized in the chromatin (*i.e.*, the extra-nucleolar part of the nucleus). The incorporation by nuclei of anucleolate tissue did not show any localization, but was subnormal and remarkably similar to the incorporation by the chromatin of the control tissue. The simplest interpretation of this result is that nucleolar incorporation is entirely into ribosomal RNA, and that all other incorporation into RNA (mostly into soluble RNA) occurs in the chromatin of both the anucleolate mutants and controls.



TEXT-FIGURE 2.—Incorporation of ^3H -uridine by *Xenopus* embryos. Ordinate: mean grain count per nucleus standardized to 80 days' exposure; abscissa: time of incubation in hours. +--+ Control total nucleus; ●—● control chromatin (excluding nucleolus); ○—○ anucleolate mutant nucleus.

Anucleolate nuclei usually possess a variable number of RNA-containing "blobs" (2, 11). No such blobs could be resolved through the autoradiograph film, nor were they indicated by any accumulation of grains on the film. It is still possible that these blobs do represent accumulations of soluble RNA and that they might be revealed as such by specific labeling during the methylation of soluble RNA (12-14). Axial fragments of *Xenopus* embryos were accordingly incubated in methyl- ^{14}C -methionine at 50 $\mu\text{C}/\text{ml}$ for 35 minutes and prepared for autoradiography as described before. This resulted in a fairly uniform incorporation of label (into

both RNA and protein) over the nuclei and cytoplasm, with no striking difference between anucleolate and control tissues. A pretreatment with puromycin at 350 $\mu\text{g}/\text{ml}$ for 35 minutes greatly reduced this incorporation, presumably by inhibiting protein synthesis. After puromycin treatment, control tissues showed the greatest intensity of grains over nucleoli; anucleolate tissues showed a reduced but still uniform pattern of labeling. A preliminary analysis of the RNA by sucrose density-gradient centrifugation indicates that the soluble RNA of anucleolate mutants is methylated to the same extent as the soluble RNA of controls, but that the ribosomal RNA of control embryos is also methylated. The labeled protein fractions from such extracts suggest that the rate of protein synthesis in anucleolate mutants at stage 42, when their development is visibly retarded, has been reduced only to half that of the control larvae.

The results obtained so far from *Xenopus* thus indicate that the synthesis of ribosomal RNA on a nucleolar organizer is an essential component of nucleolar formation. We have no definite evidence of a nucleolar function in *Xenopus*, although ribosomal RNA may be methylated there. Soluble RNA can be synthesized in the absence of the nucleolus and probably is normally synthesized independently of the nucleolus. A system of RNA methylation exists independently of the nucleolus, which may be capable of methylating ribosomal as well as soluble RNA.

RESUMEN

Se ha estudiado la incorporación de uridina H^3 y metil- C^{14} -metionina en el músculo somático de fragmentos axiales de embriones anucleolados y controles de *Xenopus*.

La incorporación de la uridina H^3 se restringe al ARN nuclear durante una incubación de hasta 3 horas. Los núcleos de tipo salvaje y los heterocigóticos muestran un reparto de incorporación (conteo de granos) de un tercio en los nucleolos y dos tercios en la región de la cromatina. Los núcleos de los mutantes anucleolados incorporan, sin ninguna localización evidente, una cantidad equivalente a la cromatina de tipo salvaje.

La metil- C^{14} -metionina se incorpora tanto en la proteína como en el ARN. El pretratamiento con puromicina reduce esta incorporación (presumiblemente del componente proteico) dando por resultado una marcación uniforme, aparte de mayores conteos en los nucleolos.

REFERENCES

- (1) WALLACE, H.: The development of anucleolate embryos of *Xenopus laevis*. *J Embryol Exp Morph* 8: 405-413, 1960.
- (2) ———: Cytological and biochemical studies of anucleolate *Xenopus* larvae. *Quart J Mic Sci* 103: 25-35, 1962.
- (3) BRISTOW, D. A., and DEUCHAR, E. M.: Changes in nucleic acid concentration during the development of *Xenopus laevis* embryos. *Exp Cell Res* 35: 580-589, 1964.
- (4) DEUCHAR, E. M., and BRISTOW, D. A.: Changing base ratios in RNA of developing tissues of *Xenopus laevis*. *Nature (London)* 205: 1321-1322, 1965.

- (5) BROWN, D. D., and LITTON, E.: RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. *J Molec Biol* 8: 669-687, 1964.
- (6) ———: Variation in the synthesis of stable RNA's during oogenesis and development of *Xenopus laevis*. *J Molec Biol* 8: 688-695, 1964.
- (7) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (8) NIEUWKOOP, P. D., and FABER, J.: Normal Table of *Xenopus laevis* (Daudin). Amsterdam, North-Holland Publishing Co.
- (9) BIRNSTIEL, M. L., WALLACE, H., SIRLIN, J. L., and FISCHBERG, M.: Localization of the ribosomal DNA complements in the nucleolar organizer region of *Xenopus laevis*. *Nat Cancer Monogr* 23: 431-447, 1966.
- (10) WALLACE, H., and BIRNSTIEL, M. L.: Ribosomal cistrons and the nucleolar organizer. *Biochim Biophys Acta* 114: 296-310, 1966.
- (11) ESPER, H., and BARR, H. J.: A study of the developmental cytology of a mutation affecting nucleoli in *Xenopus* embryos. *Develop Biol* 10: 105-121, 1964.
- (12) BIRNSTIEL, M. L., FLEISSNER, E., and BOREK, E.: Nucleolus: A center of RNA methylation. *Science* 142: 1577-1580, 1963.
- (13) BIRNSTIEL, M. L., SIRLIN, J. L., and JACOB, J.: The nucleolus: A site of transfer ribonucleic acid synthesis. *Biochem J* 94: 10-11P, 1965.
- (14) BOREK, E., and SRINIVASAN, P. R.: Alteration of the macromolecular structure of nucleic acids by transmethylolation. In *Transmethylation and Methionine Biosynthesis* (Shapiro, S. K., and Schlenk, F., eds.). Chicago, Univ Chicago Press, 1965, pp 115-137.

DISCUSSION

Brown: There are preformed maternal ribosomes present in the homozygous mutants that survive throughout their limited development. In the methylated methionine experiment are these methylated during development of the embryos?

Wallace: I do not find any methyl labeling of preformed ribosomal RNA (rRNA) from the oocyte, and, of course, none in the anucleolate mutant because it hasn't any recently synthesized rRNA. We do find it in the controls though, or I think we do.

Penman: I think we should be cautious about calling the RNA that labels, and is associated with chromatin, messenger, or else specify what we mean by it. Because, although this is RNA that is DNA-like and turns over rapidly, it apparently doesn't come into the cytoplasm, at least in our system.

Wallace: I used the term, "disperse RNA," once and used the term, "messenger RNA," once. I was speaking loosely, but I think we can communicate freely here. There is no harm in speaking loosely until we definitely say we want to be precise. When one is looking at silver grains arising from a chemical which may have been converted from the original labeled compound, then I think that one's results tend to be rather loose to begin with.

Perry: I want to make a comment that goes along with Dr. Penman's question and your reply. As you probably know, we have noted a similar situation in animal cells after administration of low doses of actinomycin D, a treatment which essentially makes them into sort of anucleolate cells (Perry, *Proc Nat Acad Sci USA* 48: 2179-2186, 1962). Now we have done hybridization tests (Perry, Srinivasan, and Kelley, *Science* 145: 504-507, 1964) which showed that the RNA that continues to be synthesized after low-dose treatment with actinomycin has a very high hybridization efficiency, the type of efficiency for hybridization that one usually ascribes to messenger RNA (mRNA). Whether it is called informational RNA or DNA-like RNA, it fulfills one of the currently accepted criteria for messenger. Whether it

ever goes to the cytoplasm and carries out a messenger function is a little bit beyond the technology that we have today. I'll put the question to Dr. Penman. Can you tell me what the evidence is that this RNA doesn't go to the cytoplasm? Can you demonstrate this under normal conditions where actinomycin is not added?

Penman: I think this can be concluded from two observations. One is related to the kinetics with which this RNA is labeled. There is as much in HeLa cells—a rapidly growing cell—as there is ribosomal precursor RNA, which is what you have also found. If one looks at the cytoplasmic fraction from HeLa cells, one can identify mRNA in it either by sedimentation or from the fact that the mRNA is all associated with the polyribosomes. Now this cytoplasmic mRNA never achieves the amount of labeling that the material in the nucleus achieves. In other words, you can label for a relatively long time and you never see this material move to the cytoplasm. In addition, it is very actinomycin-sensitive, *i.e.*, it apparently decays quite quickly in contrast to polyribosome-associated mRNA which decays slowly. This does not necessarily mean that it isn't going to the cytoplasm, but its behavior in actinomycin is different from cytoplasmic mRNA.

Feinendegen: Did you observe in your anucleolate mutant any nonrandom distribution of the grains over the chromatin portion different from that observed in the presence of the nucleolus?

Wallace: No, this is a further failure in our autoradiographs. As I have said, on one hand we were hoping that when the rRNA synthesis was abolished by the mutation, we could find the sites of synthesis of transfer RNA. The second thing, and one which I personally hoped would yield the same answer, was what are these RNA-containing bodies, bodies such as Dr. McClintock found and Dr. Swift has mentioned, and how do these come about? Are they sites of synthesis or are they collections from elsewhere? One would naturally expect the autoradiograms to show this. However, we did not find any accumulation of grains, and because of the thickness of the film and probably imperfect staining through the film, we could not resolve the RNA-containing bodies. This is very disappointing because these bodies intrigue me. Are they normal components of the nucleolus which, when rRNA is not made, are not collected into a nucleolus? For example, in this mutant and in other instances where rRNA isn't synthesized, all the necessary precursors are present to stimulate the synthesis of other kinds of RNA wherever there is a template. So these accumulations, blobs as I have called them, may well be larger than the equivalent bodies and/or accumulations of material in normal cells that have a nucleolus.

Localization of the Ribosomal DNA Complements in the Nucleolar Organizer Region of *Xenopus laevis*^{1, 2}

M. L. BIRNSTIEL,³ H. WALLACE,⁴ J. L. SIRLIN, and M. FISCHBERG,^{5, 6} *Institute of Animal Genetics, Edinburgh, Scotland, and University of Geneva, Geneva, Switzerland*

SUMMARY

When ribosomal RNA is annealed *in vitro* with denatured *Xenopus* DNA, 28S and 18S RNA combine with 0.04–0.07 and 0.025–0.04% of the DNA. The RNA-DNA duplexes detected are formed specifically between the ribosomal RNA and its complementary DNA on the basis of their ribonuclease resistance, high GC content, considerable thermostability against dissociation, and their buoyant density on a CsCl gradient. The saturation levels for ribosomal RNA indicate that there are some 500–800 ribosomal DNA complements for each of the 28S and 18S RNA's for the haploid genome of the wild-type cell. In the anucleolate, homozygous mutant there are only a few, if any, ribosomal cistrons, while the heterozygote contains only half the number present in the wild type. This linear reduction in proportion to the dosage of the mutation, evident in number of

nucleoli, number of nucleolar organizers, and number of ribosomal cistrons, establishes a direct causal relationship between these entities and suggests that the ribosomal DNA complements are confined very likely to the nucleolar organizer region and not scattered throughout the chromosomes. It is believed that owing to the grouping of the ribosomal DNA complements on the chromosome in large polycistronic clusters, fragmentation of the DNA during extraction permits the isolation of DNA molecules of considerable length which hybridize with 28S ribosomal RNA, and have a GC content equal to that of 28S ribosomal RNA. The mass of this DNA fraction approximates that predicted by the hybridization experiments and is linearly reduced by the mutation.—*Nat Cancer Inst Monogr* 23: 431–447, 1966.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This investigation was supported by the British Medical Research Council and, in part, by the Damon Runyon Fund for Cancer Research (DRG-579).

³ My attendance at this Symposium was made possible by the generosity of the Royal Society of London and The Wellcome Trust.

⁴ Present address: Biology Division, Amherst College, Amherst, Mass.

⁵ Department of Zoology, University of Geneva.

⁶ We wish to thank Professor Waddington for his interest and support and Dr. J. O. Bishop and Dr. K. Jones for many thoughtful discussions. We are grateful to J. Spiers and I. Purdom for their excellent assistance throughout this work.

A MUTATION affecting the nucleolar number in *Xenopus laevis* has been described by Elsdale, Fischberg, and Smith (1). The normal ($2n$) toad contains two nucleoli per diploid cell, the heterozygous mutant ($1n$) possesses one (1-4), and the anucleolate homozygous mutant ($0n$) has no typical nucleolus (4-6). The mutation is lethal in the homozygous condition. The mating of two phenotypically normal heterozygotes yields the three genotypes $2n$, $1n$, and $0n$ in a ratio of 1:2:1 (2) characteristic for a single (recessive) Mendelian factor.

The nucleolus usually arises at a well-defined locus on the chromosome (7-9), a secondary constriction which has been termed "the nucleolar organizer" (7). In the heterozygous *Xenopus*, a secondary constriction of one chromosome has been deleted by the mutation while the allelic chromosome remains intact. The proportionality of the nucleoli and of secondary constrictions forcibly suggests that the chromosomal segment affected by the mutation is the nucleolar organizer (10).

The anucleolate mutants do not synthesize detectable amounts of ribosomal RNA (11). However, the heterozygotes synthesize as much ribosomal RNA as the wild type; all genotypes, including the homozygous mutant tadpoles, synthesize messenger RNA and soluble RNA (11).

The absence of both the nucleolus and ribosomal synthesis underlines in a most spectacular way the relationship between these two entities (11), a connection which is well documented by biochemical data (13-17) and reiterated and corroborated at this Symposium.

Two previous attempts to establish by cell fractionation that the "nucleolar" DNA contains the genetic information for ribosomal synthesis have yielded ambiguous results (17, 18). The mutant of *Xenopus laevis* enables us to show more convincingly that the ribosomal cistrons are indeed present in that part of the chromosomes which directs the formation of the nucleolus.

In this paper we confirm Ritossa and Spiegelman's finding (19) that the ribosomal DNA complements are confined to the nucleolar organizer. We demonstrate further that the ribosomal cistrons are very likely present in large polycistrons which give rise to a distinct satellite DNA at 1.723 g/cm^3 in the *Xenopus* wild-type DNA. This minor DNA fraction comprises some 0.2% of the total DNA as predicted by hybridization data and is absent in the anucleolate homozygous mutant.

MATERIAL AND METHODS

Preparation of erythrocyte DNA of high molecular weight.—Toads were decapitated and the blood drained into ice-cold M NaCl , 0.01 M Tris $\text{pH } 7.5$, 0.01 M EDTA , and 1% mercaptoethanol under continuous agitation (10-25 ml / toad). Blood collected in this manner from 25-50 toads was made up to 1% with sodium lauryl sulfate, maintained at 60°C for 10-15 minutes, and chilled to room temperature. After adding one-tenth volume of saturated Tris solution ($\text{pH } 8.2$) and one-fourth volume

of 5 M Na ClO₄ (20) the DNA was deproteinized by shaking it with an equal volume of chloroform-phenol, 1:1. The suspension was cleared by centrifugation, the DNA was spooled and redissolved in 0.1 SSC (20). The crude DNA solution was made up to 1 M NaCl, incubated first with deoxyribonuclease-free ribonuclease (50 µg/ml) for 3 hours, then with Pronase (200 µg/ml) for 6–12 hours at 37 C. The DNA was once more deproteinized, spooled, and stored at –20 C. In some experiments the DNA received a second treatment with both ribonuclease and Pronase. In order that it might be used in the analytical centrifuge, the DNA was exhaustively dialyzed against 0.1 SSC. DNA from tadpoles of the three genotypes were prepared as reported previously (21).

Prefractionation of the DNA and formation of RNA-DNA hybrids.—Prior to hybridization with 28S RNA, DNA was fractionated in some experiments by equilibrium density centrifugation on a CsCl gradient (mean density: 1.720 g/cm³) in the rotor (SW 39) of the Spinco centrifuge Model L2 (Beckman Spinco Division, Palo Alto, Calif.) for 72 hours at 31,000 rpm and 20 C at DNA input of 150–250 µg. Two-drop fractions were collected, dialyzed exhaustively against 0.1 SSC, and the optical density of each fraction was determined at 260 mµ. Each sample was made up to 100 µg DNA by the addition of *Bacillus subtilis* DNA, annealed to 0.5–1.0 µg 28S ribosomal C¹⁴-RNA (21), and assayed for RNA-DNA hybrid formation according to Nygaard and Hall (22).

Unfractionated, total DNA was annealed to 28S or 18S ribosomal C¹⁴-RNA and the RNA-DNA hybrids were isolated as described earlier (21).

Analytical centrifugation.—DNA samples were brought to a density of 1.720 g/cm³ by the addition of CsCl and centrifuged at 44,770 rpm, 20 C in the A_n rotor of the Spinco Model E (Beckman) for 26–48 hours. The photographic records were converted to graphic traces in a Joyce-Loebl densitometer Mark IIIC (Gateshead 11, England). Denatured *Pseudomonas aeruginosa*, *B. subtilis*, or T₄ DNA's with buoyant densities of 1.740, 1.703 (23), and 1.700 (24) g/cm³, respectively, were used as markers.

Radioactivity determination.—Radioactivity was determined in a Packard Tri-Carb Scintillation Counter (Packard Instruments International, Zurich, Switzerland) as before (21). Samples were usually counted in multiples of 60 or 600 seconds and are recorded as such in the text-figures. Alternatively, the C¹⁴ radioactivity was measured in a Flow Betamat (Isotope Development Laboratories, East Kilbride, Scotland). In this instance, counting time was registered in multiples of 1,000 seconds. The C¹⁴-ribosomal RNA [prepared by pulse-chase labeling of newly hatched wild-type tadpoles with Na₂¹⁴CO₃ under aseptic conditions and subsequently repeated fractionations on sucrose gradients (21)] possessed a specific activity of 7,800–11,000 counts/60 seconds and 19,000–28,000 counts/1,000 seconds.

All other methods and materials have been described previously (21).

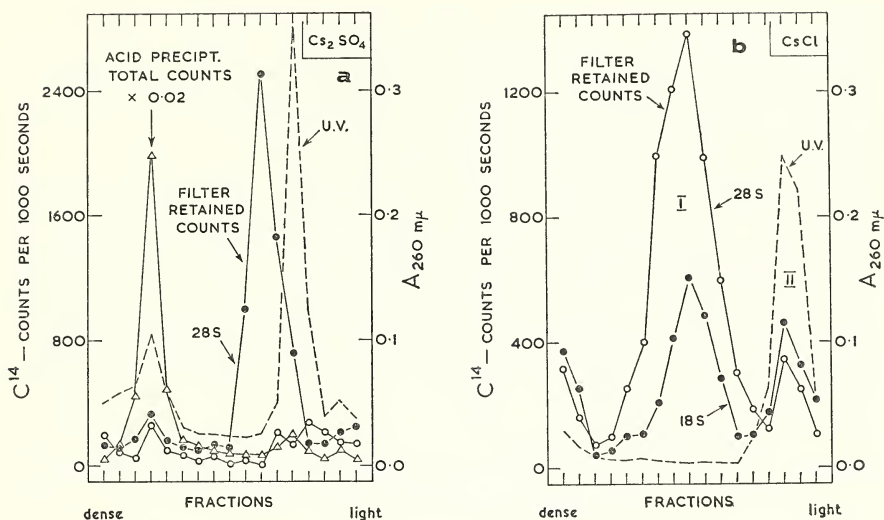
RESULTS

Ribosomal RNA-DNA Duplex Formation

Purified 28S ribosomal C^{14} -RNA was incubated with denatured DNA under conditions suitable for the formation of artificial RNA-DNA hybrids [(21); see also text-figure 1, *a* and *b*]. When the reaction products were centrifuged on a CsCl or Cs₂SO₄ gradient, the radioactivity appeared predominantly in two regions of the gradient, toward the bottom of the gradient as free RNA and toward the top as RNA accompanying the denatured DNA (text-fig. 1, *a*). A third component, intermediate in density between RNA and DNA, was revealed when the gradient fractions were disaggregated and passed through membrane filters in order to eliminate free unhybridized RNA and to select for RNA-DNA hybrids (text-fig. 1, *a* and *b*). This component is judged to be a true RNA-DNA hybrid on the basis of its high buoyant density, its filter retention, its ribonuclease resistance (21), and the fact that it is only formed with denatured, homologous DNA (text-fig. 1, *a*). That this component represents duplexes specifically between ribosomal (28S) RNA and its complementary DNA is suggested by the following evidence:

- 1) The hybridized RNA has a GC content (62% by radioactivity) similar to that of the input RNA (63% by radioactivity) (21). Contamination by messenger RNA-DNA hybrids would have led to a shift in GC content toward that of the DNA (40% GC).
- 2) The hybrid melts at high temperature and over a narrow temperature range (21). Both these features are irreconcilable with a large contamination by messenger RNA-DNA hybrids (25).
- 3) The marked buoyant density shift of the hybrid is characteristic for ribosomal RNA-DNA hybrids and is believed to be a consequence of the polycistronic arrangement of the ribosomal DNA complements (26).

Radioactivity in the region of the free RNA (text-fig. 1, *a*) is virtually abolished by the filtration procedure (22). Some radioactivity is retained in the DNA band (peak II in text-fig. 1, *b*) which, at normal RNA inputs (0.5–8 μ g/RNA/100 μ g denatured DNA), represents a small fraction compared to the RNA-DNA hybrid fractions. The material of peak II may represent, in part, spurious RNA-DNA duplex formation since it is less resistant (45%) to ribonuclease than a true RNA-DNA hybrid (80%) and since some radioactivity is recovered within regions even with denatured heterologous DNA (21). The GC content of the retained RNA, 52% by radioactivity, is shifted toward that of messenger RNA. Furthermore, unlike the ribosomal RNA-DNA hybrid which saturates at low levels of RNA, the radioactivity retained by the major DNA band increases linearly with RNA input. Both these features suggest (21) that messenger RNA may also contribute to the radioactivity recovered at the DNA band, as unremoved contamination despite a prolonged chase and exhaustive purification of the ribosomal RNA.



TEXT-FIGURE 1.—Distribution of the products of artificial RNA-DNA hybridization. Eight μg 28S ribosomal C^{14} -RNA of wild-type *Xenopus* was annealed to 100 μg denatured (or native) wild-type DNA (from feeding tadpoles; mol wt 10^7) at 70 C for 3 hours in 2 ml of $2 \times SSC$ and fractionated on a Cs_2SO_4 (a) or $CsCl$ (b) gradient. Six drops were collected, by piercing the bottom of the tube, and diluted by addition of 2 ml of $2 \times SSC$; the RNA and DNA distribution were determined by ultraviolet absorption at 260 $m\mu$. Aliquots were withdrawn for the determination of total TCA precipitable counts (a). Each gradient fraction was then diluted to 40 ml, incubated for 15–30 minutes at 70 C (18), cooled, and filtered through Millipore HA filters.

a: Fractionation on a Cs_2SO_4 gradient. Δ : total (5%) TCA precipitable counts. These values were reduced by a factor of 50 to accommodate them on the graph. \bullet : filter retained hybrids: RNA annealed with denatured DNA. The ribosomal RNA-DNA hybrid was recovered at densities between the bulk of the denatured DNA and the free RNA. \circ : Filter retained counts: RNA annealed with native DNA. No hybrid was recovered.

b: Fractionation on a $CsCl$ gradient. The conditions for annealing and disaggregation were the same as in a. \circ : Filter retained counts of 28S ribosomal C^{14} -RNA (8 μg) annealed with denatured DNA (100 μg). \bullet : Filter retained counts of 18S ribosomal C^{14} -RNA (5 μg) annealed to denatured DNA (100 μg). Peak I represents the true ribosomal RNA-DNA hybrid. Peak II represents some messenger RNA-DNA hybrid contamination and background noise (see text).

Selection of the high density hybrid discriminates successfully against both spurious RNA-DNA interaction and messenger contamination and has become standard procedure in the determination of the ribosomal RNA-DNA hybrids. Working with low RNA inputs, so as to obtain a linear response of hybrid formation with 28S RNA, and studying the partition of the hybrid between density-displaced hybrid and DNA-bound duplexes, we find that the number of ribosomal cistrons which reside in the DNA band and might go undetected by our discriminating techniques is

only a negligible minority. Our approach should therefore provide an adequate procedure for the measurement of ribosomal DNA complements. CsCl gradients are usually used for the centrifugation, since CsCl gradients are shallow and allow complete separation between high density hybrid and radioactivity bound in the DNA band.

Both 28S and 18S ribosomal RNA form high density hybrids (text-fig. 1, *b*). From the radioactivity in the hybrids, one may calculate that at saturation (text-fig. 2, *b*) the 28S RNA occupies about 0.04–0.07% and the 18S RNA about 0.025–0.04% of the DNA (21). These values appear to be additive (21). We conclude that the 28S and 18S components of ribosomal RNA in *Xenopus* differ in base sequence, annealing to and presumably derived from different DNA stretches which together occupy some 0.1% of the genome.

Hybrid Formation Between Ribosomal RNA and DNA From the Three Genotypes (*2n*, *1n*, and *0n*)

Samples of denatured DNA of the three genotypes were incubated with either 28S ribosomal C¹⁴-RNA or with 28S and 18S ribosomal C¹⁴-RNA with the following results:

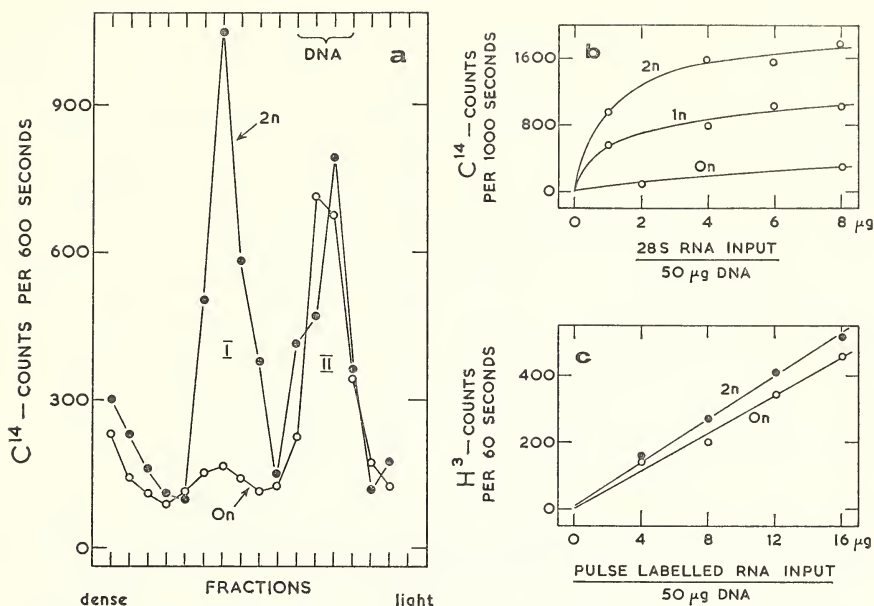
On DNA anneals very poorly to ribosomal 28S RNA. The radioactivity recovered in the high density range is negligible. To step up the sensitivity and to detect ribosomal RNA species which might possibly be poorly represented, experiments with excessive 28S ribosomal RNA inputs were carried out. As expected, these levels of RNA induced considerable accumulation of radioactivity in the DNA band which, however, does not derive from ribosomal RNA-DNA hybrids (*see above*), but the radioactivity in the high density range remains small (text-fig. 2, *a*).

If we score the high density hybrid, *1n* DNA anneals at levels intermediate between that of *0n* and *2n* DNA (text-fig. 2, *b*). In 7 sets of experiments, the saturation value of *1n* DNA annealing with 28S ribosomal RNA was on the average 57% of the *2n* control. Similarly, *1n* DNA gave rise to only half the high density hybrids compared to *2n* DNA when a combination of 28S and 18S or metabolically stable total RNA was the RNA used in the reaction.

These results suggest that the mutation measurably reduces the number of ribosomal cistrons. This reduction is linear and affects the DNA complements for both ribosomal RNA subunits. Both *2n* and *0n* DNA combine with pulse-labeled (messenger) RNA equally well (text-fig. 2, *c*), showing that the failure of the *0n* DNA to combine with ribosomal RNA is a specific defect.

A Component of *Xenopus* DNA Base-Complementary to 28S Ribosomal RNA

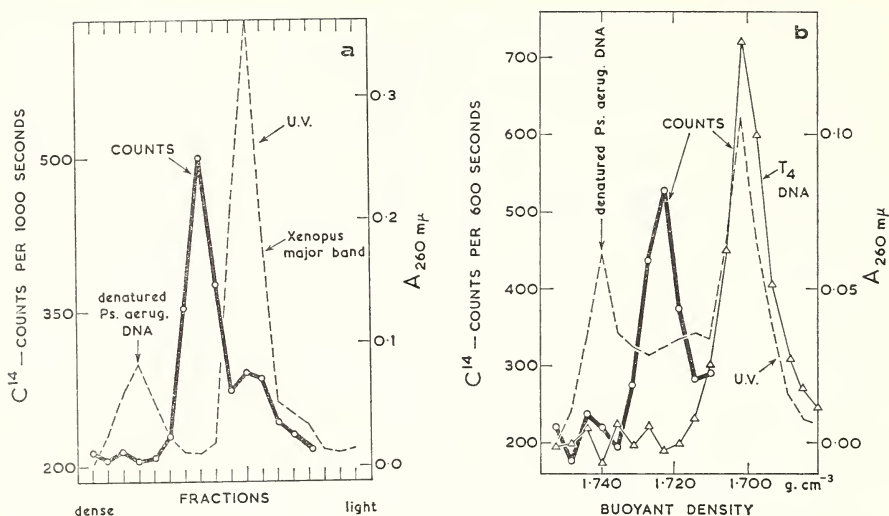
Since the ribosomal 28S RNA of *Xenopus* has a high GC content of about 65% (27), the ribosomal DNA complements must necessarily



TEXT-FIGURE 2.—Hybrid formation between ribosomal RNA or “messenger” RNA and DNA from the three genotypes of *Xenopus*. *a*: Twenty μg 28S ribosomal RNA was annealed to either $2n$ or $0n$ as in text-figure 1, *a*. Fractionation on a CsCl gradient reveals that $0n$ DNA produces only negligible high density hybrids (peak I), unlike $2n$ DNA. *b*: Saturation curves for annealing of 28S ribosomal RNA with $2n$, $1n$, and $0n$ DNA. Only the high density hybrids have been scored. Contrary to pulse-labeled RNA, ribosomal RNA saturates the relevant DNA stretches at relatively low RNA levels. *c*: Hybrid formation between pulse-labeled “messenger” RNA and DNA is similar in both $0n$ and $2n$ DNA preparations. Pulse-labeled RNA was prepared by incubating explants of tadpoles (stage 45) with $100 \mu c$ uridine- H^3 /ml ($2 \times 10^3 \mu c/\mu mole$) for 45 minutes at room temperature and subsequent extraction with hot phenol and detergent (12).

contain a similar high portion of these bases, some 25% higher than DNA as a whole. If these cistrons are linked up together in large clusters, DNA preparations of moderate molecular weight should contain DNA fragments consisting predominantly of ribosomal DNA complements.

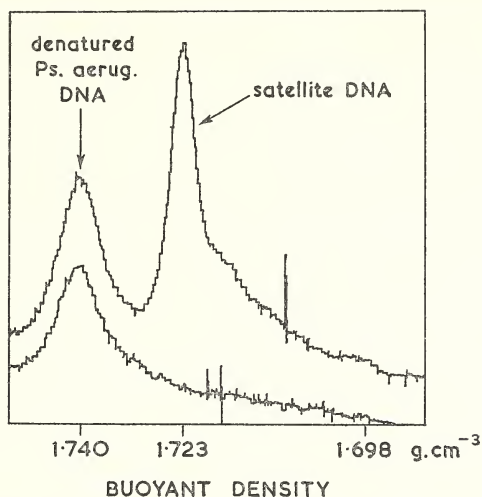
To determine whether these fragments exist, native *Xenopus* wild-type DNA molecules were first fractionated according to their GC content by CsCl gradient centrifugation. Fully denatured *Ps. aeruginosa* DNA (1.740 g/cm^3) was added as a marker and appeared as a second peak on the gradient below the *Xenopus* DNA, which has an average buoyant density of $1.698\text{--}1.700 \text{ g/cm}^3$. When the individual DNA fractions of the gradient are tested for their ability to form 28S RNA-DNA hybrids (see Methods), one finds that the main activity is distributed in a region containing little ultraviolet-absorbing material, intermediate between the denatured *Ps. aeruginosa* DNA and the main peak (text-fig. 3, *a*). The hybridization activity of these fractions, especially in view of their minute



TEXT-FIGURE 3.—Detection of a DNA component base-complementary to 28S RNA. *a*: *Xenopus* erythrocyte DNA (mol wt 2×10^7) was fractionated by a first CsCl density gradient centrifugation and each DNA fraction annealed with ribosomal RNA as described in Methods. The *Xenopus* DNA complementary to *Xenopus* 28S RNA is present mainly in a region intermediate between denatured *Ps. aeruginosa* DNA and the major *Xenopus* DNA mass. *b*: The DNA fractions, which had previously (*a*) been shown to be complementary to 28S RNA, were collected from 3–6 gradients and redistributed on a CsCl gradient by a second centrifugation. DNA fractions possessing a buoyant density of 1.722–1.724 g/cm³ annealed to 28S ribosomal C¹⁴-RNA. T₄ DNA that serves as a marker and is also radioactive prevents the accurate measurement of the hybridization in the lighter fractions. DNA fractions of *Xenopus* of lesser buoyant densities are relatively inert, as shown in *a*.

mass, must be very high. Analytical centrifugation of the DNA fractions contained in this region of the gradient revealed a major component banding at 1.723 ± 0.001 g/cm³, well displaced from the marker DNA and free from any DNA of the major band (1.698 g/cm³) (text-fig. 4). There is an additional minor contribution of *Xenopus* DNA at a density of about 1.715 g/cm³ visible as a slight shoulder on the light side of the 1.723 DNA.

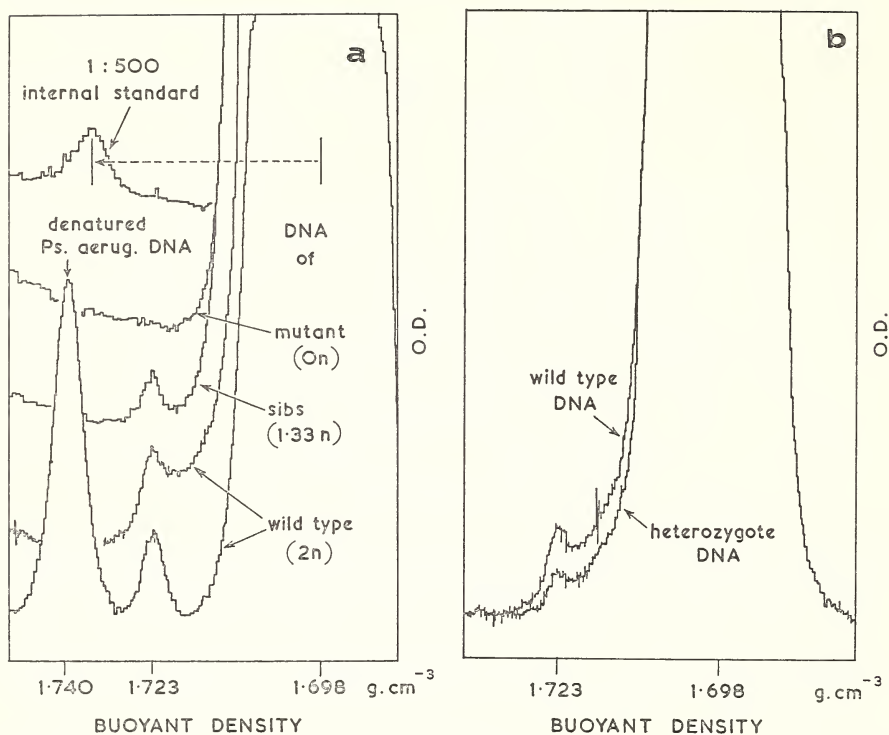
The 1.723 satellite may be observed in the analytical centrifuge without prior enrichment provided the DNA is of sufficiently high molecular weight and used in amounts 100-fold greater than those usually employed for analytical centrifugation (text-fig. 5, *a*). In addition to the satellite at 1.723 g/cm³, a second less distinct component is found at 1.715 g/cm³. The 1.723 satellite has been detected in all *Xenopus* wild-type DNA's so far investigated, *i.e.*, DNA from hatching, feeding, and metamorphosing tadpoles and erythrocytes of adult females. From comparison with standard dilutions of the same DNA solutions (run simultaneously in side wedge cells), it is determined that this satellite represents some



TEXT-FIGURE 4.—Analysis of the DNA complementary to 28S RNA in the analytical centrifuge. The gradient fractions, exhibiting great hybridization affinity for 28S ribosomal C^{14} -RNA (text-fig. 3, *a*), were analyzed in the analytical centrifuge. Fully denatured *Ps. aeruginosa* DNA (1.740 g/cm^3) served as a density marker. Upper curve: *Xenopus* DNA with the *Ps. aeruginosa* marker; lower tracing: denatured *Ps. aeruginosa* DNA alone. The tracings have been aligned relative to the density marker (see text).

0.15–0.2% of the total DNA. A comparison of the DNA of the three genotypes reveals that the heterozygous DNA contains only half of the satellite band when compared with wild-type DNA (text-fig. 5, *b*). At identical input the homozygous mutant DNA appears devoid of this satellite, while it is readily demonstrable in DNA extracted in the identical manner from phenotypically normal sibs of the same mating (text-fig. 5, *a*) and at the same developmental stage.

In tests for the complexing ability of the individual fractions, we usually overloaded the CsCl gradients with DNA. These high DNA inputs make the recovery of equal fractions along the gradient difficult, especially in the region containing the highly viscous major DNA band, and may also cause some deviation from the linearity of the gradients. To mitigate these possible effects an experiment was performed in which DNA was first fractionated on a CsCl gradient, and the relevant regions were collected and redistributed by a second centrifugation to equilibrium. In this experiment, denatured *Ps. aeruginosa* DNA and C^{14} -T₄ DNA were used as markers (text-fig. 3, *b*). The T₄ DNA served as a second reference point since the first centrifugation might have selected a heavy fraction from the major *Xenopus* DNA band and rendered it unsuitable as a marker. From the position of the hybridization activity in the gradient in relation to these two markers, the DNA, base-complementary to 28S RNA, may be



TEXT-FIGURE 5.—Tracings of photographic records obtained from analytical centrifugation of DNA samples. *a*: The CsCl gradient (at a mean density of 1.720 g/cm^3) contained the following amounts of DNA (mol. wt. $10\text{--}50 \times 10^6$): homozygous mutant: $87 \mu\text{g}$; phenotypically normal sibs of the same matings: $69 \mu\text{g}$; wild type (without marker): $102 \mu\text{g}$; wild type with denatured *Ps. aeruginosa* as a density marker: $95 \mu\text{g}$. As an internal standard for the determination of the satellite mass, a 500-fold dilution of the wild-type DNA (without marker) was used in parallel experiments. The major DNA band (1.698 g/cm^3) shows a considerable spread owing to the fact that it is present in 100-fold excess. The tracings have been displaced vertically to facilitate comparison. Similarly, the internal standard has been shifted to a region of high density. A satellite at 1.723 g/cm^3 is present in all preparations at high DNA input, with the exception of the homozygous mutant DNA. A further *Xenopus* component at 1.715 g/cm^3 also becomes apparent, especially in DNA samples of a molecular weight of about 30×10^6 (not shown). *b*: CsCl gradient centrifugation of homozygous and heterozygous DNA. The DNA input was $105 \mu\text{g}$ in each case. The samples were run simultaneously in the analytical centrifuge which was equipped with an alternator for two-cell operation. The heterozygous DNA contains only half the 1.723 satellite mass. The DNA material at 1.715 g/cm^3 is probably also reduced in the mutant.

placed at a buoyant density of $1.722\text{--}1.724 \text{ g/cm}^3$ in good agreement with the buoyant density of the 1.723 DNA band previously detected in the analytical centrifuge. Analysis of an aliquot of this density range now reveals that the 1.723 DNA is the only DNA component present in these fractions (not shown).

DISCUSSION

If one assumes a molecular weight for the two ribosomal components of 1.6 and 0.63×10^6 (28), there are in each diploid *Xenopus* wild-type nucleus ($6 \mu\text{g}$ DNA) some 1,000–1,600 stretches of DNA complementary to 28S ribosomal RNA and 1,000–2,000 DNA complements of the 18S ribosomal RNA. If one assumes a one strand copy of DNA, these ribosomal DNA complements would constitute 0.08–0.1% and 0.05–0.08% (together 0.13–0.22%) of the total cellular DNA. This percentage determined by artificial RNA-DNA hybridization is similar to that measured in bacteria (29), pea seedlings (17), and fruit flies (19) by the same means.

In good agreement with the above values, we find that the DNA satellite banding in CsCl at a density of 1.723 g/cm^3 contains some 0.15–0.2% of the total DNA. This band possesses the following attributes consistent with the presence of 28S ribosomal DNA complements.

- 1) The GC content of this band calculated from the buoyant density (30–32) is 65%, identical to that of 28S ribosomal RNA (27).

- 2) It hybridizes with 28S ribosomal RNA. Interestingly enough, *Ps. aeruginosa* DNA with a GC content of some 66% does not anneal to *Xenopus* 28S ribosomal RNA (see text-fig. 3, a and b). This demonstrates that successful hybridization not only requires the appropriate GC content, but necessitates the correct base sequence register.

- 3) The mass of the satellite approximates that anticipated from the hybridization experiments. The sharpness of this band suggests that the DNA molecules in the satellite band are relatively homogeneous with respect to GC content. This correlates with the previous finding that the ribosomal RNA-DNA hybrids melt over a narrow temperature range (21). The satellite is present even in DNA with a molecular weight in excess of 5×10^7 (though perhaps in lesser quantity). If the average molecular weight is a valid measure of the length of the high GC fragments, this would indicate the existence of very extended clusters of ribosomal DNA complements. An independent determination of molecular weight of the satellite DNA from the band width indicates (33) the presence of DNA stretches of a molecular weight of $\geq 10^7$, which could contain enough information for several 28S ribosomal RNA's (≥ 3) or at least one 45S ribosomal precursor molecule (12).

- 4) The satellite mass in the three genotypes exhibits a linear reduction with the dosage of the mutation and thus parallels the results obtained by the artificial RNA-DNA hybridization experiments.

This linear dosage effect also makes it unlikely that the satellite band represents DNA derived from contamination by bacteria, viruses, or mitochondrial DNA. Moreover, the satellite is present in DNA from erythrocytes which are presumably free from bacteria.

It is concluded that the 1.723 DNA satellite includes 28S ribosomal DNA complements. Whether it accommodates 18S ribosomal RNA cistrons as well is not known.

Both techniques, RNA-DNA hybridization and direct measurement of the 1.723 satellite, indicate that the anucleolate mutant of *Xenopus* contains only a few, if any, ribosomal cistrons. This finding eliminates the hypothesis that the mutation merely inactivated the ribosomal cistrons through a defective operator mutation (11). Moreover, the absence of the satellite in the mutant suggests that the ribosomal DNA complements have been physically removed and not merely rendered inactive for hybrid formation by some chemical alteration.

We conclude that in the anucleolate mutant a chromosomal deletion eliminating all or most of the ribosomal DNA complements, has occurred. The removal of the ribosomal cistrons by one chromosomal event seems only possible if they are massed together and the disappearance of 0.2% of the cellular DNA, corresponding to some 4% of one chromosome, might well result in a visible alteration of the chromosome structure.

The number of nucleoli, the number of nucleolar organizers, and the number of ribosomal DNA complements all show a linear reduction with the dosage of the mutation. From the evidence available so far, we propose as the simplest hypothesis that the deletion has occurred in that part of the chromosome which is reported to have been removed by the mutations (10), namely, in or near the nucleolar organizer region (*see also* page 563), and that consequently the ribosomal cistrons in *Xenopus*, like those in *Drosophila* (19), are to be found at or near that segment of the chromosome which gives rise to the nucleolus.

In bacteria (34, 35) the ribosomal cistrons are also massed on a short segment of the bacterial chromosome [probably in close proximity to the genes that code for transfer RNA and the ribosomal structural proteins (35)]. A clustering of the bacterial ribosomal DNA complements had been previously suggested by Yankofsky and Spiegelman (26) from the large displacement of the ribosomal RNA-DNA hybrids from the density of denatured DNA. On this basis polycistrons are also demonstrable in *Xenopus*. The relative homogeneity and considerable molecular weight of the high GC-DNA fragments also support the contention that ribosomal DNA complements are arranged in polycistronic clusters. This is also consistent with the grouping of a large number of ribosomal cistrons on a small segment of the chromosome of *Xenopus laevis*.

Note added in proof: *Xenopus* wild-type DNA sedimenting at 105S or faster possesses no 1.723 satellite. Satellite DNA seems to be "knitted into" the bulk of the DNA. As the DNA is sheared the satellite band appears and in DNA of 50-60S some 50% is liberated. Assuming random breakage of the DNA molecules, these data suggest that the high G-C stretches are of considerable length corresponding to a molecular weight of the order of 10^8 . As expected, DNA preparations of the anucleolate mutant exhibit no 1.723 satellite at any level of fragmentation. Shearing the DNA below 20S does not appreciably augment the satellite mass which represents at maximum 0.21% of the total DNA.

RESUMEN

Cuando el ARN ribosómico es incubado in vitro con ADN desnaturalizado de *Xenopus*, el ARN 28S y el 18S se combinan al máximo con el 0.07 y 0.04% del ADN. Las duplas ARN-ADN detectadas se forman específicamente entre el ARN ribosómico y su ADN complementario sobre la base de su resistencia a la ARNasa, alto contenido en GC, su considerable termoestabilidad a la disociación y su densidad de flotación sobre un gradiente de CsCl. Los niveles de saturación para el ARN ribosómico indican que hay algunos complementos de ADN ribosómicos 500-800 para cada uno de los ARN 28S y 18S por genomio haploide de la célula de tipo salvaje. En el mutante homocigótico anucleolado hay sólo unos pocos, si hay alguno, cistrones ribosómicos, mientras que el heterocigótico contiene solamente la mitad del número presente en el tipo salvaje. Esta reducción linear proporcional a la dosis de la mutación, evidente en número de nucleolos, número de organizadores nucleolares y número de cistrones ribosómicos, establece una relación causal directa entre estas entidades y sugiere que los complementos del ADN ribosómico están confinados muy probablemente en la región del organizador nucleolar, y no dispersados a lo largo de los cromosomas. Se piensa que debido al agrupamiento de los complementos del ADN ribosómico sobre el cromosoma en grandes grupos policistrónicos, la fragmentación del ADN durante la extracción permite el aislamiento de las moléculas de ADN de largo considerable que se hibridan con el ARN ribosómico 28S y tienen un contenido en GC igual al del ARN ribosómico 28S. La masa de esta fracción de ADN se aproxima a la predicha mediante experimentos de hibridación y está reducida linealmente por la mutación.

REFERENCES

- (1) ELSDALE, T. R., FISCHBERG, M., and SMITH, S.: A mutation that reduces nucleolar number in *Xenopus laevis*. *Exp Cell Res* 14: 642-643, 1958.
- (2) FISCHBERG, M., and WALLACE, H.: A mutation which reduces nucleolar number in *Xenopus laevis*. In *The Cell Nucleus* (Mitchell, J. S., ed.). London, Butterworth, 1960, pp 30-34.
- (3) BARR, H. J., and ESPER, H.: Nucleolar size in cells of *Xenopus laevis* in relation to nucleolar competition. *Exp Cell Res* 31: 211-214, 1963.
- (4) WALLACE, H.: Cytological and biochemical studies of anucleolate *Xenopus laevis*. *Quart J Micr Sci* 103: 25-35, 1962.
- (5) ESPER, H., and BARR, H. J.: A study of the developmental cytology of a mutation affecting nucleoli in *Xenopus* embryos. *Develop Biol* 10: 105-121, 1964.
- (6) JONES, K. W.: The role of the nucleolus in the formation of ribosomes. *J Ultrastruct Res* 13: 257-262, 1965.
- (7) MCCLINTOCK, B.: The relationship of a particular chromosomal element to the development of the nucleoli in *Zea mays*. *Z Zellforsch* 21: 294-328, 1934.
- (8) HEITZ, E.: Die Ursache der gesetzmässigen Zahl, Lage, Form und Grösse pflanzlicher Nucleolen. *Planta* 12: 775-844, 1931.
- (9) BEERMANN, W.: Der Nukleolus als lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.
- (10) KAHN, J.: The nucleolar organizer in the mitotic chromosome complement of *Xenopus laevis*. *Quart J Micr Sci* 103: 407-409, 1962.
- (11) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc Nat Acad Sci USA* 51: 139-146, 1964.
- (12) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4s RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (13) EDSTRÖM, J.-E.: Composition of ribonucleic acid from various parts of spider oocytes. *J Biophys Biochem Cytol* 8: 47-51, 1960.

- (14) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosomes. *Biochim Biophys Acta* 76: 454-462, 1963.
- (15) COMB, D. C., BROWN, R., and KATZ, S.: The nuclear DNA and RNA components of the aquatic fungus *Blastocladiella emersonii*. *J Molec Biol* 8: 781-789, 1964.
- (16) VINCENT, W.: The nucleolus. In *Genetics Today, Proceed XI Intern Congr of Genetics*, 1963. London, Pergamon Press, 1965, II, pp 342-358.
- (17) CHIPCHASE, M. I. H., and BIRNSTIEL, M. L.: On the nature of nucleolar RNA. *Proc Nat Acad Sci USA* 50: 1101-1107, 1963.
- (18) McCONKEY, E. H., and HOPKINS, J. W.: The relationship of the nucleolus to the synthesis of ribosomal RNA in HELA cells. *Proc Nat Acad Sci USA* 51: 1197-1204, 1964.
- (19) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (20) MARMUR, J.: A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Molec Biol* 3: 208-218, 1961.
- (21) WALLACE, H., and BIRNSTIEL, M. L.: Ribosomal cistrons and the nucleolar organizer. *Biochim Biophys Acta* 114, 296-310, 1966.
- (22) NYGAARD, A. P., and HALL, B. D.: A method for the detection of RNA-DNA complexes. *Biophys Biochem Res Commun* 12: 98-104, 1963.
- (23) GANESAN, A. T., and LEDERBERG, J.: Physical and biological studies on transforming DNA. *J Molec Biol* 9: 683-695, 1964.
- (24) ERIKSON, R. L., and SZYBALSKI, W.: The Cs_2SO_4 equilibrium density gradient and its application for the study of T-even phage DNA: glucosylation and replication. *Virology* 22: 111-124, 1964.
- (25) BOLTON, E. T., and MCCARTHY, B. J.: Fractionation of complementary RNA. *J Molec Biol* 8: 201-209, 1964.
- (26) YANKOFSKY, S. A., and SPIEGELMAN, S.: The identification of the ribosomal RNA cistron by sequence complementarity. II. Saturation of and competitive interaction at the RNA cistron. *Proc Nat Acad Sci USA* 48: 1466-1472, 1962.
- (27) BROWN, D. D., and LITINA, E.: RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. *J Molec Biol* 8: 669-687, 1964.
- (28) STAEHELIN, T. H., WETTSTEIN, F. O., OURA, H., and NOLL, H.: Determination of the coding ratio based on molecular weight of messenger ribonucleic acid associated with ergosomes of different aggregate size. *Nature (London)* 201: 264-270, 1964.
- (29) YANKOFSKY, S. A., and SPIEGELMAN, S.: Distinct cistrons for the two ribosomal RNA components. *Proc Nat Acad Sci USA* 49: 538-544, 1963.
- (30) SUEOKA, N., MARMUR, J., and DOTY, P.: II. Dependence of the density of deoxyribonucleic acids on guanine-cytosine content. *Nature (London)* 183: 1429-1431, 1959.
- (31) ROLFE, R., and MESELSON, M.: The relative homogeneity of microbial DNA. *Proc Nat Acad Sci USA* 45: 1039-1043, 1959.
- (32) SUEOKA, N.: Variations and heterogeneity of base composition of DNA: A compilation of old and new data. *J Molec Biol* 3: 31-40, 1961.
- (33) VINOGRAD, J., and HEARST, J. E.: Equilibrium sedimentation of macromolecules and viruses in a density gradient. *Fortschr Chem Organ Naturst* 20: 373-422, 1962.
- (34) OISHI, M., and SUEOKA, N.: Location of genetic loci of ribosomal RNA on *Bacillus subtilis* chromosome. *Proc Nat Acad Sci USA* 54: 483-491, 1965.
- (35) DUBNAU, D., SMITH, I., and MARMUR, J.: Gene conservation in bacillus species. II. The location of genes concerned with the synthesis of ribosomal components and soluble RNA. *Proc Nat Acad Sci USA* 54: 724-730, 1965.

DISCUSSION

Perry: A point of clarification: What did you say the $\rho = 1.715$ band corresponds to?

Birnstiel: We have not determined this yet.

Perry: Is it just a satellite that you pick up by this technique as sort of a by-product of isolating that region of the gradient and rerunning it?

Birnstiel: That's right.

Ritossa: How do you calculate the percentage of hybridized ribosomal RNA (rRNA)? Do you include the part that bands with the bulk DNA?

Birnstiel: No. When I talked about amounts of rRNA-DNA hybrids, it refers in all cases to the high density hybrid only.

Ritossa: What is the molecular weight of your DNA preparations?

Birnstiel: It is usually between 5 and 20 million. But we have done hybridization experiments to observe the high density shifts with DNA with molecular weights as high as 50 million.

Ritossa: I think this is an important point. Since you have a DNA of such a molecular weight you might expect that only a part of the DNA complementary to rRNA is effectively split out of the nonrelevant DNA. I do not think you have adequate evidence to conclude that none of the hybrid RNA which bands with the bulk DNA is ribosomal. If there is continuity in the chromosomal DNA, a part of the DNA not complementary to rRNA could be adjacent to that which is complementary to it. In this case you might expect to have a certain amount of rRNA properly hybridized but staying with the bulk DNA. This would have an influence on the calculation of the saturation level, especially when you plot number of nucleolar organizers versus saturation level. You would not have a line passing straight through the origin, but shifting a little from it.

Birnstiel: This is a point certainly deserving further investigation. From the limited information we have so far, we think the fraction of ribosomal cistrons remaining in the major DNA band upon hybridization is negligible and thus is not detected. When we prefractionate DNA prior to annealing, we find that the ribosomal cistrons do, in fact, sort themselves out fairly well and there is little or no trailing of hybridized material from the high density region. This is further evidence that either the changeovers between ribosomal and nonrelevant DNA are infrequent or that there exist predetermined breaking points where the ribosomal cistrons are knitted into the chromosomal DNA.

Vincent: In your original saturation curves, you gave values of a thousand counts/10,000 seconds. What level of reproducibility does this provide?

Birnstiel: The counts reported in 1,000 units were measured on an ideal low-background counter with a background of 3 counts/minute.

Busch: I am unfamiliar with the base composition of the 18S and 28S rRNA of *Xenopus*. Is it identical to that of mammalian 18S and 28S rRNA? In mammalian ribosomes the 18S rRNA is substantially more AU-rich than the 28S rRNA.

Birnstiel: According to Dr. Brown (Brown and Littna, *J Molec Biol* 8: 669-687, 1964), the 28S rRNA contains some 65% and the 18S rRNA some 56-60% GC. The two species thus appear to be very distinct from one another.

Mandel: I should like to ask a very naive question. Is the organizer a DNA cistron or operon for rRNA?

Birnstiel: This is a very difficult question. From the *Xenopus* work, as well as from Dr. Ritossa's work, we can only say that the organizer region contains the ribosomal cistrons. Whether the concept of the nucleolar organizer finds its physical basis in these cistrons, or their operator genes, or some other genes, I do not know.

Barr: Dr. Birnstiel, before your presentation there was a question about whether the *Xenopus* story involves a physical deletion or not, and you certainly have cleared that up very beautifully. There is still a question, however, about the relationship of the deletion you have demonstrated to the nucleolar organizing region. There is

no karyological information that really localizes the nucleolar organizer in *Xenopus*, such as there is for maize. It might be that there is a region, the nucleolar organizer, where nucleolar materials are put together. Among the things put together at this organizer could be rRNA which was synthesized somewhere else in the genome. So, I believe there is a slight logical confusion here. I don't think we really have the right to call a region which is complementary to rRNA the nucleolar organizer. It may be more complicated than that.

Birnstiel: All we can say is that a deletion has occurred which eliminates the ribosomal cistrons in one step and that this deletion very likely has occurred near to or in a secondary constriction which has been previously identified as the nucleolar organizer (Kahn, *Quart J Micr Sci* 103: 407-409, 1962).

Barr: The presence or absence of a secondary constriction, as seen in a metaphase chromosome, is not necessarily equivalent to the presence or absence of a piece of chromosomal material. It is very likely that the constriction is formed because the nucleolus is formed there. And if for some reason or other the nucleolus is not formed, then there is no secondary constriction. That is, the absence of a secondary constriction is not diagnostic of a deletion. An example of this, which Dr. Esper and I (*Develop Biol* 10: 105-121, 1964), have discussed in a paper relative to the *Xenopus* problem is Dr. McClintock's work in maize where the nucleolar organizer is clearly mapped (McClintock, *Z Zellforsch* 21: 294-328, 1934). In this case the deletion is found in a totally different and separate region from the nucleolar organizer. When this deletion is present, the nucleolar organizer does not function and no constriction is found.

McClintock: The constriction observed at metaphase reflects the mode of formation of the nucleolus at telophase and the manner of its disappearance at late prophase. Increase in size of the nucleolus at telophase is associated with separation of the chromosome into two longitudinal parts at the region of the nucleolus organizer. As the nucleolus commences to regress in size in late prophase, these two parts approach each other, but usually they do not come into direct contact before the nucleolus is completely released from its association with the chromosome. The gap between the two parts of the chromosome remains and appears at metaphase as a "constriction." A nucleolus organizer in one chromosome, known to be fully functional under certain circumstances, may not be able to compete successfully in the same nucleus with a nucleolus organizer in another chromosome. As a consequence, it may produce either no nucleolus or only a tiny one at telophase. Thus, since the parts of this chromosome are not separated at the nucleolus organizer region during telophase, the chromosome will not exhibit a constriction at that region in the following metaphase.

Ritossa: Perhaps I missed Dr. Barr's point. As he mentioned, there can be mutations in other parts of the genome which inhibit the formation of the nucleolus. Let's assume that the cistrons for rRNA are the material basis of the organizer. Let's also suppose that the formation of the nucleolus depends on the effective formation of ribosomes (or of ribosomal precursors). In this case, if you block any component other than RNA which has to do with ribosome formation—one of the proteins, for example—you would still inhibit nucleolus formation even with the cistrons for rRNA present.

Birnstiel: Of course, our measurements, like those of Ritossa, establish correlations between the number of nucleoli, nucleolar organizers, and ribosomal cistrons, and, as such, they have certain shortcomings. In our cytological interpretations we assumed—I think correctly—that these items are strictly interdependent. One can also show that all other possible interpretations are artificial and unlikely and I believe Professor Waddington will discuss this further in his paper (p. 563).

Vincent: The problem of the nature of the nucleolus organizer is one of the more important aspects of the nucleolus which we can discuss at this Symposium. I would like to make some general comments relating to it.

First of all, I must emphasize that the nucleolus is primarily protein, and, as Dr. Ritossa has pointed out, one should be able to modify or inhibit nucleolus formation by affecting synthesis of some necessary protein. It is very important, therefore, that we keep in mind we are studying only 5 to 10% of the nucleolus when we look at its RNA. In some place or from somewhere protein arises which collects at the nucleolus. The nature of these proteins is not understood, but as I shall point out later, they most certainly are not all ribosomal proteins.

The evidence presented by Dr. Birnstiel tells us that the rRNA cistrons are localized in the nucleolar organizer region, and presumably Dr. Ritossa will demonstrate that the nucleolar organizer region can be localized at a specific region of a chromosome arm. The evidence available allows us to conclude that deletions of this area are expressed both as failure of organization of a nucleolus and as a failure to synthesize rRNA. The evidence thus far does not allow the conclusion that the synthesis of rRNA is the sole function of the nucleolar organizer region. Indeed, we need much more precise genetical analyses of this region, such as those carried out by Dr. McClintock and Dr. Pelling, before a strict definition of the nucleolar organizer in molecular terms is possible.

Pavan: Dr. Vincent has been telling us from the first day of this meeting that the starfish nucleolus is 95% protein. My point is this: Can we use that as a generalization, or are these nucleoli different from those of other organisms? Another point I would like to discuss is related to what has been said about rRNA. Although I agree with the evidence so far presented that rRNA is produced only at the nucleolar organizer region, I do not think we have enough data to say this is a universal process. For instance, in *Rhynchosciara* our results with autoradiography and the results of Lara with RNA sedimentation (unpublished) suggest that ribosomal RNA may be synthesized in different parts of the polyten chromosome of the salivary gland and not around the nucleolar organizer region only.

Ritossa: Regarding Dr. Vincent's point, I would like to ask a question about chromosome puffs and the protein at the puff level. A puff can be considered an active locus. Our view of the nucleolus as the sole site of synthesis of ribosomes is a similar concept. I would like to ask what is the proportion, if known, between the nonhistone protein which accumulates at the time of puff formation and the RNA in the puff? This ratio could be important because in order for a transcription of a stretch of 200 cistrons to go on, a certain amount of polymerase will be needed. Furthermore, if the RNA synthesized there is also transformed into rRNA there, this transformation would probably require a different enzyme. This could account for a portion of the nucleolar protein in addition to what ribosomal proteins may be present.

Brown: At this point the argument is one of semantics. This mutant has lost the secondary constriction which is usually defined as the nucleolar organizer region. Furthermore, the number of these constrictions corresponds to the number of nucleoli. Now if this mutation were not a single deletion, then the argument against the nucleolar organizer being the site of the structural genes for rRNA would be more meaningful. If someone still wants to argue that the nucleolar organizer is another chromosome locus which indirectly directs rRNA synthesis by genes located elsewhere, he must explain the heterozygote. According to this reasoning, the heterozygote should have not one but two nucleoli.

Waddington: Dr. Birnstiel, are the data you presented on the hybridizing ability of the anucleolate mutant related entirely to 28S rRNA? You said that 18S rRNA can hybridize with the normal diploid, but you gave no data about 18S rRNA hybridization with the mutant.

Birnstiel: We have not annealed 18S rRNA by itself to mutant DNA, but only mixtures of 28S and 18S rRNA. These experiments show the same linear reduction with the dosage of the mutation as with 28S rRNA alone. Since we know that 28S and 18S rRNA anneal additively, it would appear that the mutation affects the 18S rRNA cistrons in an identical fashion.

On the Chromosomal Distribution of DNA Complementary to Ribosomal and Soluble RNA^{1, 2}

F. M. RITOSSA,³ K. C. ATWOOD, D. L. LINDSLEY,⁴
and S. SPIEGELMAN, *Department of Microbiology, University of Illinois, Urbana, Illinois*

SUMMARY

Molecular hybridization experiments with labeled ribosomal RNA (rRNA) and DNA from *Drosophila* stocks carrying different doses of the nucleolar organizer (NO) showed that the wild-type genome saturates at 0.27% of the DNA, which indicates that for each of the two rRNA components approximately 130 complementary sites exist per haploid set. The proportions of the DNA complementary to rRNA in the different stocks correspond to those predicted from their genetic constitutions and the assumption that all the DNA complements of rRNA are confined to the NO region. Competitive hybridization with tritium-labeled 28S rRNA and P³²-labeled 18S RNA established that these two ribosomal components of *Drosophila melanogaster* are generated by their own distinct DNA templates. Analogous questions were answered with respect to transfer RNA (tRNA). The saturation values obtained indicate that approximately 0.018% of the DNA of *D. melanogaster* is complementary to tRNA. This number leads to

an approximately 15-fold redundancy for each of the approximately 60 tRNA species. Hybridization, carried out with DNA from the stocks carrying the various doses of the NO region, established that tDNA cannot be detected in that region. Chromosomal or nonchromosomal location of the rRNA cistrons was examined by means of saturation hybridization with DNA derived from tissues differing in the extent to which they are engaged in rRNA synthesis. These included sperm, erythrocytes, embryo, kidney, and liver of the chicken. The saturation plateaus of the DNA from these various tissues were essentially identical within experimental error. Certain properties of the *bobbed* mutation, which is located close to the NO, led to the hypothesis that the *bobbed* region contains rDNA. A number of *bobbed* mutants were tested and it was found that their saturation plateaus were significantly lower than normal.—Nat Cancer Inst Monogr 23: 449–472, 1966.

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² The investigations reported here were supported by grants GB-2169, GB-2700, GB-2699 from the National Science Foundation, by Public Health Service research grant CA-01094 from the National Cancer Institute, and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

³ On leave from the International Laboratory of Genetics and Biophysics, Naples, Italy.

⁴ Biology Division of the Oak Ridge National Laboratory, Oak Ridge, Tenn.

THE INVOLVEMENT of the nucleolus in cell growth and protein synthesis (1, 2) acquired a more precise molecular interpretation with the suggestion that it was concerned with ribosome formation (3-6).

The possibility that the nucleolus is merely the location for the assembly of ribosomes from RNA and protein has been postulated (7) as opposed to the view that the nucleolus is the actual site of ribosomal RNA (rRNA) synthesis (3). One approach to the solution of this problem has been to test the homology to rRNA of the DNA associated with the nucleolus by RNA-DNA hybridization (8, 9). However, when DNA obtained from isolated nucleoli was used, contradictory results were obtained (7, 10).

Another question on the role of the nucleolus concerns its participation in amino acid transfer RNA (tRNA) synthesis. tRNA has indeed been reported in the nucleolus (11) and RNA methylating enzymes are represented there in considerable amounts (12, 13), which suggests that the nucleolus is the site of tRNA formation (14). However, nucleolar RNA synthesis can be inhibited with practically no interference with tRNA synthesis (3), and a mutant of *Xenopus laevis* exists that is unable to form nucleoli but nevertheless retains complete ability to synthesize tRNA (5).

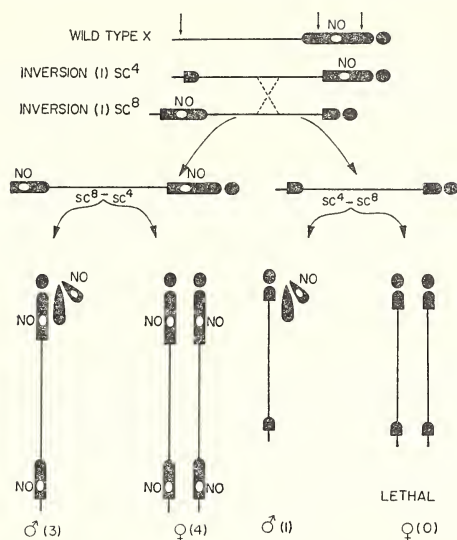
RNA-DNA hybridization combined with genetic manipulation seemed to offer us the best opportunity for deciding whether complements to both rRNA and tRNA are in the chromosomal DNA that is invariably associated with nucleolus formation. (For convenience, we will refer to the DNA segments complementary to tRNA and rRNA as tDNA and rDNA.) Crossing-over between $\text{In}(1)sc^4$ and $\text{In}(1)sc^8$ [or the similar $\text{In}(1)sc^{81}$] of *Drosophila melanogaster* leads to the formation of X chromosomes either deficient or duplicated for a short piece of heterochromatin encompassing the nucleolar organizer.

This device allows the insertion of different doses of the region of interest into substantially the same genome (15). The sensitivity reached in the newly improved hybridization technique (16) is now such that the presence or absence of even small amounts of complementary DNA can be ascertained with adequate confidence.

The following sections summarize our attempts to resolve some of the questions raised by the combined use of DNA-RNA hybridizations and recombinational genetics.

LOCALIZATION OF RIBOSOMAL RNA CISTRONS (rDNA)

Wild X and Y chromosomes of *D. melanogaster* each possess one nucleolar organizer (NO), which is localized in the proximal, heterochromatic part of the X and on the short arm of the Y, which is also heterochromatic (17). Stocks of *D. melanogaster* possessing various doses of NO regions can be obtained according to the scheme outlined in text-



TEXT-FIGURE 1.—Origin and construction of the stocks. See text for description.

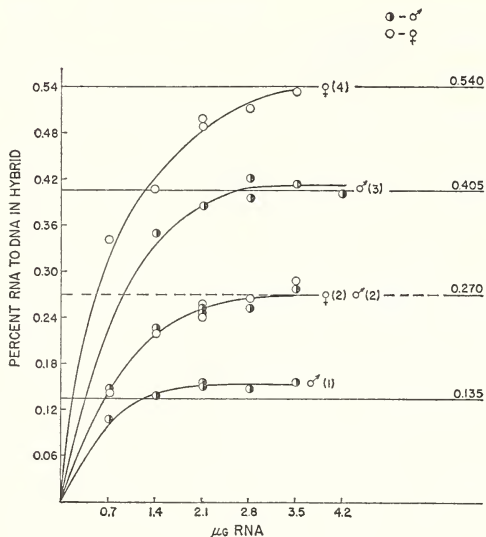
fig. 1. The two different inversions of the X chromosome possess almost the same left point of breakage. The right point of breakage of one is situated to the left of the NO and of the other to the right of NO.

Crossing-over in a female carrying both of these inversions gives rise to one deleted (sc^4sc^8) and one duplicated (sc^8sc^4) chromosome with respect to the region included between the right point of breakage of the two original inversions. We will call this piece of chromosome the sc^8 - sc^4 region. The stocks used in our analysis have the following relevant constitution with respect to the sex chromosomes; the number of NO regions is given in parentheses.

♂ (1) : $In(1)sc^{4L}sc^{8R}/Y$; ♂ (2), ♀ (2) : X/Y ; X/X ; ♂ (3) : $In(1)sc^{81L}sc^{4R}/Y$; ♀ (4) : $In(1)sc^{81L}sc^{4R}/In(1)sc^{81L}sc^{4R}$.

Since males and females had to be compared in our analysis, we first determined whether they carried the same amount of DNA complementary to ribosomal RNA. This was the case when X and Y chromosomes from wild populations were compared. The curve for wild type (text-fig. 2) compares male and female DNA separately and clearly shows that X and Y contribute equally to the total DNA complementary to rRNA. It is therefore possible to compare saturation values of hybridization tests without regard to the sex of the individuals.

The results of the hybridization between labeled rRNA and DNA derived from the various stocks described are shown in text-figure 2 and the saturation plateaus plotted against dosage of NO are given in text-figure 3. It is clear that the saturation values are directly proportional to the number of nucleolar organizers.

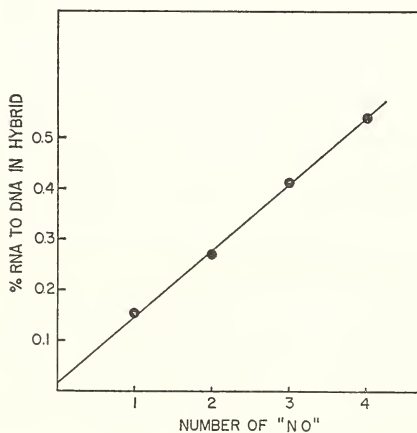


TEXT-FIGURE 2.—Saturation levels of DNA containing different dosages of nucleolar organizer (NO) regions with rRNA. Dosage of NO is indicated by the number in parentheses. *Dotted horizontal line* at 0.27 is assumed to be a correct estimate for a dosage of 2, and *solid horizontal lines* represent predicted plateaus for dosages of 1, 3, and 4, respectively. Numerical values of the plateaus are given on right. All hybridizations and subsequent ribonuclease and washing treatments were carried out as previously described (6).

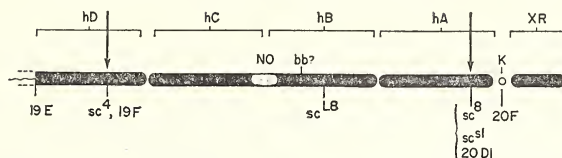
The stocks we used do not differ only in the number of nucleolar organizers. As may be seen from text-figure 4, the region deleted or repeated to achieve various doses of NO in the X chromosome contains the entire sc^s-sc^4 region, which includes hB and hC elements and a part of the hA and hD elements of the proximal heterochromatin of the X (17).

The sc^s-sc^4 region represents 25–30% of the mitotic X chromosome. If the X chromosome is assumed to contain approximately 20% of the DNA of haploid set, the sc^s-sc^4 region in turn constitutes about 5% of a haploid amount of DNA. The proportionality between the amount of rRNA hybridized and the number of NO regions led us (6) to conclude that practically all the information for the synthesis of rRNA is in this region.

To provide the reader with the magnitudes involved, we briefly describe the method used for detecting and correcting for nonspecific binding



TEXT-FIGURE 3.—Relationship between number of nucleolar organizers and percentage of rRNA to DNA in hybrid at saturation.



TEXT-FIGURE 4.—Schematic representation of the heterochromatic part of the X chromosome of *Drosophila melanogaster*. [Redrawn from Cooper (17).]

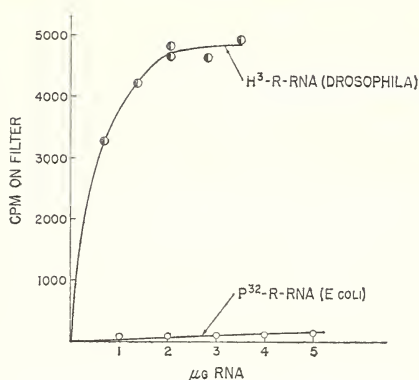
("noise") of RNA. *Drosophila* DNA is annealed with H^3 -labeled *Drosophila* rRNA in the presence of an equal amount of *Escherichia coli* or *Bacillus megaterium* rRNA labeled with P^{32} . The amount of P^{32} -RNA that survives the ribonuclease treatment and subsequent washing steps is subtracted from the H^3 -labeled rRNA. A numerical example of the procedure is detailed in table 1 and text-figure 5.

TABLE 1.—Details of a saturation experiment

Incubations were carried out in 4 ml of $2 \times$ SSC at 65 C. The indicated amounts of ♂(1) DNA were prefixed on the membrane filters. The specific activity of the H^3 -RNA of *Drosophila* was 77,285 cpm/ μ g and that of P^{32} -RNA of *Escherichia coli* was 9200 cpm/ μ g. Removal of irrelevant RNA by washing and ribonuclease treatment as described. H^3 -“noise” is calculated from the finally observed P^{32} -counts corrected for difference in specific activities. All recorded counts are corrected for background. Numbers in the first 3 columns refer to nucleic acid added in μ g.

<i>Drosophila</i>		<i>E. coli</i> P^{32} -RNA	cpm- H^3	cpm- P^{32}	H^3 -noise	% of genome
DNA	H^3 -RNA					
36.5.....	0.7	1	3284	10	83	0.113
37.3.....	1.4	2	4283	11	91	0.145
37.1.....	2.1	3	4838	15	124	0.164
37.3.....	2.1	3	4680	16	132	0.157
37.3.....	2.8	4	4609	20	166	0.154
37.3.....	3.5	5	4923	23	190	0.164

The fact that the points of text-figure 3 extrapolate so well to the origin offers evidence for the following conclusions: 1) Our method of hybridization and detection effectively eliminates “noise” due to nonspecific adsorption of RNA. 2) Our rRNA preparations are not detectably contaminated with messenger RNA (mRNA). 3) No significant proportion of rDNA is found outside the sc^8 - sc^4 region.



TEXT-FIGURE 5.—Saturation value by use of DNA from ♂ (1). H³-counts represent hybrid RNA, P³²-counts are “noise.” See table 1 and text for explanation.

REDUNDANCY OF THE RIBOSOMAL CISTRONS

The percentage of rRNA to DNA in the hybrid at saturation is about 0.27% in the wild type. The haploid genome of *D. melanogaster* contains between 0.2 and 1×10^{-12} g (18, 19) of DNA. The lower figure corresponds to 1.2×10^{11} daltons. By use of the entire rRNA fraction, the molecular weight of the DNA complementary to rRNA is therefore 3.2×10^8 daltons.

Since the molecular weight of the combined 28S and 18S particles is approximately 2.4×10^6 , it is evident that the haploid genome contains information equivalent in sequence length to 130 stretches of each of the two (18S and 28S) rRNA components.

DISTINCT CISTRONS FOR 28S AND 18S RNA COMPONENTS

It was shown (20) by competitive hybridizations with suitably labeled RNA that the 16S and 23S molecules of rRNA in *B. megaterium* are complementary to different DNA sequences. Since recent data (21) have raised some doubts on this conclusion, experiments were performed (22) to determine whether in *Drosophila* the 18S molecule does or does not derive its primary structure from the same templates used for the synthesis of 28S molecules.

As may be seen from table 2, base composition alone does not permit a decision since the 18S and 28S are very similar, a situation not unlike that found in bacteria. A variety of experiments can be performed to decide whether the sequences occupiable by 18S RNA can also accept 28S RNA. In the present experiment advantage was taken of the fact that, under the conditions employed, a hybrid structure is stable (see data of table 3). It will be noted that no counts are detectably displaced from the hybrid structure during the second annealing in the presence of an excess of unlabeled RNA.

TABLE 2.—Base composition of *Drosophila melanogaster* RNA fractions

P³²-labeled 28S and 18S fractions were purified on sucrose density gradients [12 hours at 25,000 rpm in an SW 25 (Spinco head) according to the methods of Sherrer and Darnell (23)]. tRNA was purified by MAK column to release it from free P³². RNAs were digested 18 hours at 30 C in 0.35 N NaOH. Nucleotides were separated by a Dowex 1-X8 column as standardized by Hayashi and Spiegelman (24). As control, 2–3 mg cold *Escherchia coli* total RNA was used.

The base composition of the DNA was determined by equilibrium in a CsCl density gradient. *Pseudomonas aeruginosa* DNA was used as a reference.

	C	A	U(T)	G
28S.....	17.5	30.4	30.1	22.0
18S.....	17.8	28.1	31.0	23.1
tRNA.....	25.9	20.2	24.6	29.3
DNA.....	20.0	30.0	30.0	20.0

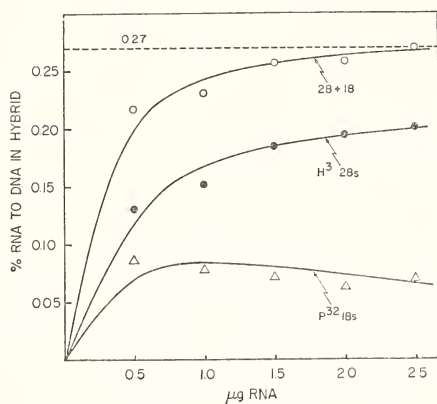
TABLE 3.—Stability of rRNA in hybrid

About 30 μ g denatured DNA from wild-type *Drosophila melanogaster* was loaded on a nitrocellulose filter as previously described (6). Two cycles of hybridization were then performed with rRNA as indicated in the table. The incubations were performed for 12 hours at 65 C. The numbers in the table represent μ g RNA per 3 ml 2 \times SSC.

After the first annealing the filters were exhaustively washed with 2 \times SSC. After the second annealing the filters were washed again with 2 \times SSC, treated with ribonuclease, washed, dried, and counted.

I° annealing	II° annealing in presence of:	cpm/100 μ g DNA
3.0 H ³ -rRNA	No RNA	12,664
3.0 "	" "	12,081
3.0 "	" "	12,907
3.0 "	10 μ g unlabeled	12,071
3.0 "	" "	12,986
3.0 "	" "	11,863

With this device, the following experiment was performed. The DNA was first saturated with H³-28S RNA and then annealed with P³²-18S. The results are shown in text-figure 6. The 28S RNA alone was unable to attain values close to the saturation plateau at concentrations where bulk RNA easily attains it (text-fig. 2). Neither does prehybridization with "saturating quantities" of 28S prevent the P³²-18S RNA from entry into the hybrid. A slight rise in H³-28S accompanied by an equivalent fall in the P³²-18S was consistent with a small but detectable contamination of the 28S by 18S-RNA. However, the sum of the two yielded a saturation curve completely comparable to that observed with bulk RNA.



TEXT-FIGURE 6.—Saturation levels of separated 28S and 18S rRNA. Denatured DNA from wild-type *Drosophila* was fixed to nitrocellulose filters. The filters were first incubated in 3 ml $2 \times$ SSC at 65 C with increasing amounts of H^3 -labeled 28S rRNA. After 12 hours of incubation, the filters were washed with $2 \times$ SSC and a second cycle of annealing was performed with P^{32} -labeled 18S rRNA in the same conditions as above. The filters were then washed with $2 \times$ SSC, incubated 1 hour at 30 C in $2 \times$ SSC containing 20 μ g/ml boiled ribonuclease, washed again, dried, and counted. The two isotopes were counted on different channels of a TRI-CARB liquid scintillation counter. 28S and 18S rRNA were previously purified through two cycles of sucrose density gradient.

The results described are in complete agreement with the conclusions of uniqueness drawn by Yankofsky and Spiegelman (20) in their study of the 23S and 16S components of *B. megaterium*. The two ribosomal RNA components of *D. melanogaster* are generated by their own distinct DNA templates.

ON THE QUESTION OF DNA COMPLEMENTARY TO AMINO ACID TRANSFER RNA (tRNA)

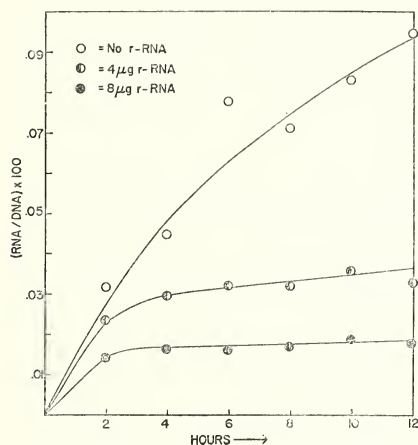
The existence of DNA complementary to amino acid tRNA has been established in the bacteria (25, 26). The availability of the *D. melanogaster* DNA in the studies just described enabled the extension of this question to higher forms (27). In particular, it permitted the performance of the necessary experiment relevant to the following two questions: 1) What proportion of the genome is complementary to tRNA? 2) Are the DNA complements of tRNA localized in the same region as those of the rRNA?

An answer to the first question is pertinent to possible interpretations of the 130-fold redundancy of the rDNA complements, since the same factors requiring redundancy in rDNA can plausibly be advanced as operative for the tDNA. The resolution of the second question has obvious implications for problems of control and parallel variation. Thus one might perhaps intuitively imagine that since both are concerned with protein synthesis a contiguous location of rDNA and tDNA would permit easier regulation of both amount (28, 29) and extent of function. In

addition, recent reports of tRNA in the nucleolus have led to the conjecture (14) that it may have a local origin.

The tRNA used in the hybridization experiments to be described were isolated on columns of methylated albumen coated on kieselguhr (MAK) and characterized in sucrose gradients as having a sedimentation coefficient of "4S." Table 2 compares the base composition of the "4S" fraction with that of the two ribosomal RNA components and the DNA of *D. melanogaster*.

Despite chemical and physical evidence of comparative purity, caution must be exercised in interpretation of hybridization data since even minor contamination can generate considerable confusion. The type of difficulty should perhaps be specifically spelled out so that the reader can assess the experiments described here and similar ones by others. If the RNA sample being tested contains a contaminant, however minor, which is complementary to a much larger segment of DNA than the RNA species being examined, no meaningful plateau can be achieved. Conversely, even extensive contamination will not generate serious difficulties if the contaminating RNA species is complementary to a much smaller DNA segment than the RNA being tested.



TEXT-FIGURE 7.—Kinetics of hybridization of tRNA from *Drosophila* with increasing amounts of cold rRNA. About 100 μ g DNA was stuck to nitrocellulose filters and incubated for increasing amounts of time at 50 C in 3 ml $2 \times$ SSC containing 1.33 μ g tRNA/ml. (O) No cold rRNA was added; (●) 4 μ g cold rRNA/ μ g tRNA was added to the incubation mixture; (⊙) 8 μ g cold rRNA was added per μ g tRNA.

With 4S RNA, the presence of even small amounts of labeled rRNA will introduce a difficulty since its plateau value is known to be high. The search for the 4S RNA plateau could thus be obscured. That this is true is shown in text-figure 7, which also demonstrates how the addition of unlabeled rRNA easily eliminates the confusion. The plateau value obtained for 4S RNA, under these circumstances, is 0.018% of the genome.

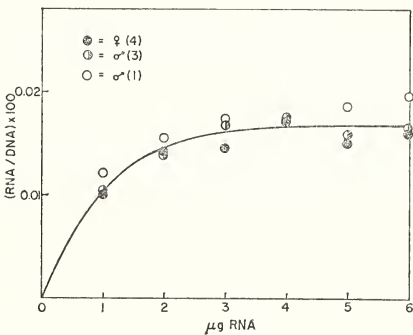
As with rRNA, it was of obvious interest to determine initially whether a difference could be detected between males and females in the amount of DNA found to be complementary to tRNA. For this purpose, DNA from wild-type males and females was tested in separate hybridization

TABLE 4.—Comparison of saturation plateaus with DNA from wild-type males and females

Saturation levels on DNA from separated, wild-type males and females. The filters were loaded with about 100 μg DNA and incubated for 12 hours at 50 C in 3 ml $2 \times \text{SSC}$ with increasing amounts of H^3 -labeled 4S RNA as indicated. For each μg 4S-RNA, 1 μg P^{32} -labeled *Escherchia coli* rRNA, and 8 μg of unlabeled rRNA from *Drosophila* were added. The calculations for “noise” and handling of the filters are as described in table 2.

γ 4S RNA	% DNA hybridized	
	σ^+	φ^+
1.....	0. 0099	0. 0082
2.....	0. 0123	0. 0115
3.....	0. 0159	0. 0141
4.....	0. 0178	0. 0189
5.....	0. 0190	0. 0163
6.....	0. 0187	0. 0209
Plateau (average of 4, 5, and 6 μg).....	0. 0185	0. 0187

experiments with results as recorded in table 4. The plateau values, 0.0185 and 0.0187, are clearly indistinguishable. Since XX and XY combinations contribute equivalently, sex can be ignored in comparing saturation values obtained with the various stocks to be tested for effects of NO dosage.



TEXT-FIGURE 8.—Saturation levels of DNA carrying different doses of nucleolar organizer regions with tRNA. DNA (70–100 μg) was fixed to nitrocellulose filters and incubated in 3 ml $2 \times \text{SSC}$ for 12 hours at 50 C in the presence of increasing amounts of H^3 -labeled tRNA. The incubation mixture contained also 1 μg P^{32} -labeled *Bacillus megatherium* rRNA/ μg tRNA and 8 μg cold rRNA from *Drosophila*/ μg tRNA. (O) φ (4); (\odot) δ (3); (\otimes) δ (1).

Text-figure 8 summarizes the results of the experiments carried out with DNA derived from flies carrying 1, 3, and 4 dosages of the NO region. It is obvious that the three types of DNA give the same plateau of about 0.018% obtained (table 4) with the two wild-type DNAs containing 2 NO dosages. These data clearly discourage localizing any significant proportion of the tRNA cistrons in the region which was deleted and duplicated in the stocks employed.

This outcome stands in striking contrast to the similar experiments with rRNA hybridization in which the rDNA content was directly proportional to the dose of NO. We conclude that, unlike the rDNA, very little—possibly none—of the tDNA is in the NO or the sc^8 - sc^4 segment. A further conclusion is justified that the X chromosome alone does not contain a preponderance of the tDNA. Finally, since each kind of tDNA is required for genetic translation, any tDNA in the Y chromosome must also be represented elsewhere.

Our results are consistent with the conclusions of Perry (3) from experiments with L cells in which partial inhibition by actinomycin D prevented synthesis of nucleolar RNA, but allowed synthesis of a 4S component at least some of which was probably tRNA, in extranucleolar parts of the nucleus. Similarly in agreement is the finding of Brown and Gurdon (5) that lethal homozygotes of the anucleolate mutant of *Xenopus laevis* can synthesize 4S RNA, but not rRNA.

MULTIPLICITY OF tDNA CISTRONS AND ITS IMPLICATIONS

We have already noted that the DNA content of the haploid *D. melanogaster* corresponds to an equivalent molecular weight of 1.2×10^{11} daltons. When this value is multiplied by the average saturation plateau of 0.018% we obtain ca. 2.2×10^7 for the molecular weight equivalent of the total tDNA. With 60 different kinds of tDNA, the average quantity of a given kind is ca. 3.7×10^5 daltons. This divided by the molecular weight of tRNA, 2.5×10^4 , gives an average of 15 templates per haploid set for the transcription of each kind of tRNA. As already suggested (6, 9, 30) for the case of rDNA, the redundancy of tDNA may be related to a requirement at certain times of a peak rate of tRNA synthesis that exceeds the maximum possible transcription rate of a single template. Concurrent transcription of an appropriate number of templates would meet such a requirement.

Note that the comparative redundancy obtained for the two types of RNA is in agreement with this interpretation. The equivalent molecular weight for one set of ribosomal RNAs is about 2×10^6 and the corresponding number for one complete set (*i.e.*, 60) of sRNAs is not very different, being 1.5×10^6 . In cells one finds, in general, 10 times as much rRNA as tRNA. Therefore, if a 130-fold redundancy of rDNA satisfies the template requirements of rRNA, a multiplicity of 13 should do for sRNA, a value not far from the 15-fold found.

The possession of multiple copies, far in excess of two for each type of tRNA, would make it difficult to employ suppressor mutations which function via mutations in tRNA anti-codons to correct corresponding codon mistakes in structural cistrons. The efficiency with which this type of suppression would operate would decrease with increasing tRNA re-

dundancy. Thus, while the tRNA type of suppression is common in the bacteria, it may be less likely to occur in higher forms.

The question will arise whether the redundancy of the rDNA and tDNA is built into the mitotic chromosomes, or instead represents local elaboration of DNA segments in some manner disproportionate to the remainder of the genome. This possibility will be discussed in a subsequent section.

Assuming for the moment that the redundancy is chromosomal, the demonstrated multiplicity of both rDNA and tDNA has a bearing on the question of multistrand chromosome models. A multistranded chromosome would have for any DNA region a redundancy, calculated by the method used here, at least as great as the number of strands the chromosome comprises. Any amount in excess of that number necessarily represents DNA segments repeated in the same strand. Thus, the multiplicity of a specific segment of DNA provides an upper limit for the strandedness of the chromosome. Since the redundancy of tDNA is much less than that of rDNA, we can conclude that the redundancy of rDNA is largely in the form of linear repeats rather than multiplicity of strands.

A POSSIBLE GENETIC INTERPRETATION OF THE tDNA CISTRONS

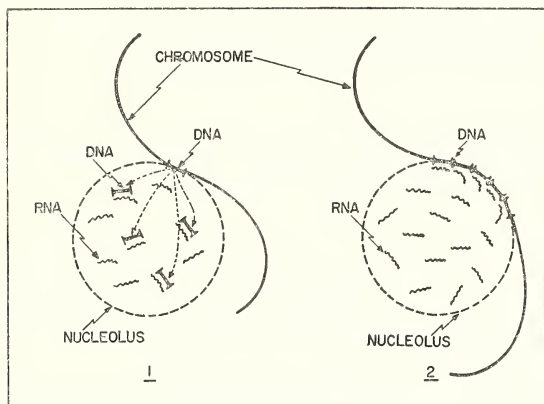
We mention here a conjecture, to be developed in detail elsewhere (31), which locates the tDNA cistrons at the genetic loci of the dominant markers known as Minutes. The *Minutes* form a phenotypically homogeneous class with an estimated number of about 55 members. Ten to 15% are in X, and the rest distributed among the autosomes. They are most frequently caused by deletions, suggesting that each locus has linear redundancy in the presence of which point mutations are largely ineffective. The 15-fold redundancy of tDNA is consistent with the foregoing interpretation of the Minutes. Further, a deletion in any one of them should lead to a qualitatively similar phenotypic effect. Finally, if they are scattered, there should be in the neighborhood of 60 such loci. The phenotype—delayed development, small bristles, homozygous lethality—is not inconsistent with a general retardation of genetic translation.

CHROMOSOMAL VERSUS NONCHROMOSOMAL MULTIPLICITY FOR rRNA CISTRONS

We have seen that there are approximately 130 cistrons for each of the rRNA types per haploid genome of *D. melanogaster*. Similar high levels of multiplicity have been obtained in plants (7), amphibia (32), and mammals (10, 21). Even the bacteria exhibit multiplicities of 5–20 (9).

The question immediately arises as to the nature of this apparent redundancy and its relation to the chromosome. If a cell requires multiple copies of a particular cistron at some stage to satisfy an elevated demand

for the corresponding RNA complements, it can, in principle, solve the problem in two different ways (text-fig. 9). In case 1, the chromosomes are presumed to possess one or a few copies of the required cistron. We assume then the existence of an independent magnification achieved via the replication of the required cistrons when needed. In case 2, the number of cistrons maximally required pre-exist as linear repeats in the chromosome. These considerations are applicable only where at least a certain amount of true redundancy of information for rRNA synthesis is required, *i.e.*, where the 130 cistrons are not all functionally differentiated.

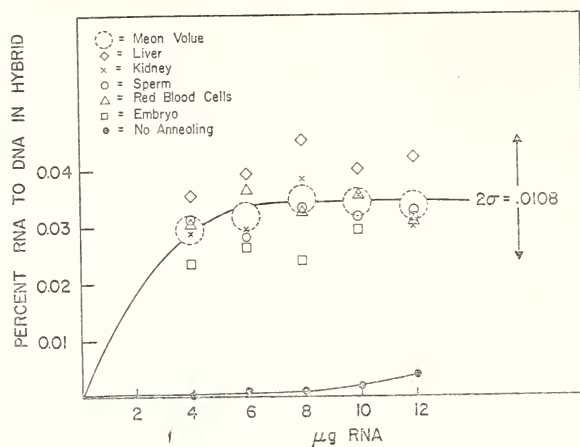


TEXT-FIGURE 9.—Possible models justifying the presence of multiplicity of cistrons for rRNA. Model 1 postulates the existence of a mechanism of magnification of one of few chromosomal cistrons. Model 2 implies the insertion of all the cistrons in the chromosome.

While the second hypothesis represents the “classical” view, suggestive evidence for the first hypothesis is to be found in reports of the existence of a metabolic DNA [review in Lima-de-Faria (33)]. In particular, the presence of DNA rings in the nucleoli of the newt oocytes (34, 35) suggests further support for this view. To decide between the two hypotheses, experiments were done with DNA from various tissues of the chicken (36).

The hypothesis of chromosomal multiplicity predicts that DNA isolated from different tissues will exhibit the same percentage hybridizable with rRNA. The alternative hypothesis would be supported if the saturation levels of the DNA from different organs varied with the tissue of origin, reflecting different intensities of rRNA synthesis.

Specifically, one might expect DNA from sperm and erythrocytes, in which no rRNA synthesis occurs, to contain smaller amounts of rDNA than the DNA from organs more or less actively engaged in rRNA synthesis, *e.g.*, embryo, kidney, and liver. As may be seen from text-figure 10 and table 5, the saturation plateaus of the DNA from all tissues were essen-



TEXT-FIGURE 10.—Saturation of DNA from different organs of the chicken with rRNA. The rRNA was from the embryo and the label used was P^{32} .

tially identical within experimental error. The percentage of rRNA to DNA in the hybrid at saturation is about 0.03. With the haploid genome size of the chicken being 1.2×10^{-12} g, one estimates about 100 rRNA cistrons per haploid genome.

The data just summarized are consistent with the existence of chromosomal multiplicity. The possibility, however, of the existence of an independent mechanism of magnification of these multiple cistrons cannot be rigorously ruled out at this stage of the investigation. Further, one

TABLE 5.—Percent rRNA bound to chicken DNA in hybrid

Quantities (70–80 μ g) of each sample of DNA were fixed to nitrocellulose filters. They were incubated for 12 hours at 65 C in 3 ml of $2 \times$ SSC containing increasing amounts of P^{32} -labeled rRNA. After ribonuclease digestion, the RNA counts were calculated as a percentage of the amount of DNA left on each filter.

There is no significance between the mean saturation values as determined by a t test. The t values are: sperm-kidney 1.933; sperm-liver 2.789; sperm-erythrocytes 1.914; sperm-embryo 0.956.

With 1% probability of error over 8 degrees of freedom $t=3.355$.

Similar figures were obtained also with H^3 -labeled rRNA.

μ g rRNA in incubation mixture	Sperm	Kidney	Liver	Erythrocytes	Embryo
4.....	0.0261	0.0269	0.0315	0.0272	0.0223
6.....	0.0291	0.0319	0.0334	0.0323	0.0263
8.....	0.0267	0.0338	0.0355	0.0330	0.0240
10.....	0.0330	0.0332	0.0483	0.0333	0.0293
12.....	0.0286	0.0353	0.0477	0.0360	0.0315
Mean.....	0.0287	0.0322	0.0393	0.0323	0.0267
Standard deviation.....	± 0.0027	± 0.0030	± 0.0081	± 0.0032	± 0.0038

can still argue that even in the case of sperm the multiple nonchromosomal copies exist. It is necessary to supplement the experiments described with other evidence, in particular genetic, before any hard inferences can be drawn.

Chromosomal multiplicity of a given cistron can in principle be achieved by "parallel" insertion of several copies of that cistron along a single chromonema (28, 29, 37, etc.) or by "serial" repetition within the chromonema (38, 39). When the number of copies per genome of two different types of cistron are known, however, a maximum figure of chromonemata per chromosome is automatically identified. It corresponds to the number of copies of the lesser of the two kinds of cistrons represented. Thus, the values obtained with tRNA and rRNA in *Drosophila* allow us to conclude that some linear multiplicity for rRNA cistrons must exist.

rRNA CISTRONS WITHIN THE sc^3 - sc^4 REGION AND THE "BOBBED" MUTATION

The sc^3 - sc^4 region represents about 25% of a mitotic X chromosome corresponding to about 5% of a haploid set of *D. melanogaster*. Since the saturation level of DNA with rRNA is 0.27%, only approximately 1/20th of this region corresponds to the segment relevant to our hybridization experiments. As we previously observed, the sc^3 - sc^4 region includes part of the hA and hD elements and the entire hB and hC elements of Cooper (text-fig. 4).

An attempt to refine the localization of the rDNA within the sc^3 - sc^4 region could make use of different inversions having their right points of breakage nearer to the NO, or of free duplications covering the different heterochromatic elements of the sc^3 - sc^4 region.

A possible approach to the problem came, however, by the use of an hypothesis concerning the mutation known as *bobbed* which has been located in the sc^3 - sc^4 region. On examination, certain correlations between the properties of *bobbed* and the known features of rDNA became apparent. We can list the information as follows:

- a) The nucleolus is the site of formation of ribosomes (or of ribosomal precursors).
- b) There is multiplicity of cistrons for rRNA and a part, at least, of these cistrons must be linearly arrayed on an "ultimate chromonema."
- c) Evidence exists for contiguity of these cistrons (32, 40).
- d) The several cistrons for rRNA, if not completely alike, are, at least, sufficiently similar to guarantee nucleolar formation and survival when subdivided (41-43).
- e) One dose of NO is sufficient to guarantee a normal phenotype.

The presence of tandem duplications provides the possibility of unequal crossing-over (44), even if the duplicated parts are somewhat different, i.e., have to be considered as a pseudoallelic series (45). We entertained the hypothesis that the *bobbed* mutants could represent deletions of cistrons for rRNA.

Following the reasoning outlined above, one would expect the *bobbed* phenotype to appear only when the deletion of the rRNA cistrons is greater

than one half the wild locus. The following known properties of *bobbed* can be marshaled as arguments in favor of this hypothesis.

1) In the X chromosome, the *bobbed* locus has been located "either to the left or right of the nucleolar organizer" (17), and the NO is associated with nucleolar formation. The "NO" is a cytological entity and in *Drosophila*, at least, it is referred to as a chromosomal constriction. The molecular basis of any kind of nucleolar formation, however, must reside in the neighborhood of this constriction.

2) A wild "allele" (and its mutations) of the *bobbed* locus is present in the short arm of the Y chromosome (46) where the NO of the Y chromosome is located (17).

3) In other species of *Drosophila* in which the NO is not exclusively localized on the sex chromosomes, the *bobbed* locus was found to be similarly displaced (47).

4) The *bobbed* mutants are recessive, i.e., the presence of a wild "allele" guarantees a wild phenotype, and the different mutants have an additive effect (48).

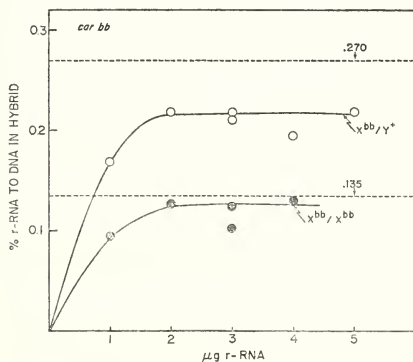
5) These mutants revert to wild type and this reversion is not always associated with the accumulation of extra Y chromosomes.

6) The *bobbed* phenotype is what one would expect for the presence of a defective machinery of protein synthesis (slow development, short bristle, low viability, and fecundity, etc.).

An experimental test of the validity of this hypothesis would examine the amount of rDNA in *bobbed* mutants by hybridization with rRNA. The theory would predict that stocks showing the *bobbed* phenotype should yield saturation plateaus less than 0.135% of the genome.

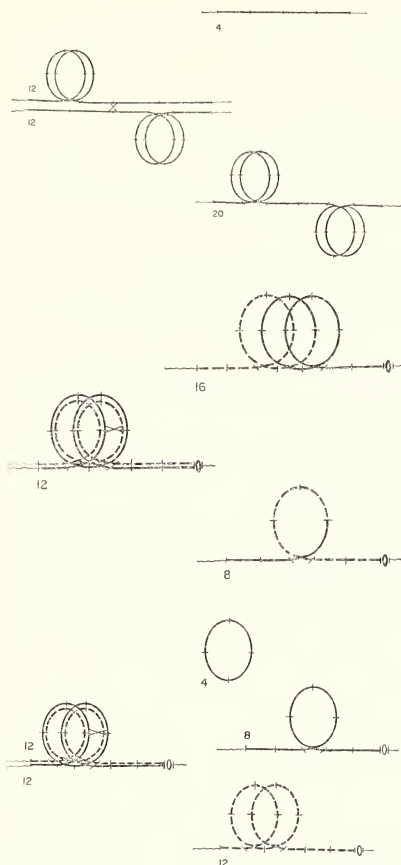
The frequent and rapid degeneration of *bobbed* stocks to wild type introduces a difficulty in the performance of these experiments. However, hybridization tests between DNA from *bobbed* and labeled wild-type rRNA showed that the *bobbed* phenotype is associated with genotypes carrying a deletion of DNA complementary to rRNA.

The results of one of these experiments are shown in text-figure 11 in which the saturation curves for a male (X^{bb}/Y , hence not *bobbed*), and a female (X^{bb}/X^{bb}) phenotypically *bobbed* are compared.



TEXT-FIGURE 11.—Saturation curves of DNA from a *bobbed* mutant and rRNA. The stock is *car bb* from Oak Ridge National Laboratory. A partial selection for *bobbed* has been done some generations before the test. Males (carrying a normal Y chromosome and an X chromosome with the *bb* mutation) and females (with both the X with the mutation) have been challenged. Two repetitions by use of female's DNA are reported.

The experiments have been done with a sufficiently large number of stocks (*car bb*; *y w bb^{ds}*; *bb¹*, all from Oak Ridge Natl. Lab.) to insure against the possibility that the *bobbed* mutation was associated with a chance deletion of rDNA. The level of saturation between DNA from



TEXT-FIGURE 12.—Diagrammatic representation of unequal crossing-over. All the corresponding chromosomal elements are paired. An alternative possibility is offered by intrachromosomal exchange (see text-figs. 13 and 14). The validity of this scheme has been experimentally shown (49). The effective existence of these mechanisms in our material can be checked and represents one of our future tasks.

TEXT-FIGURE 13.—Diagrammatic representation of intrachromosomal exchange. If the exchange occurs between an old chromatid and a new one, one of the chromatids gains cistrons at the expense of the other. [Redrawn from Peterson and Laughnan (49).]

TEXT-FIGURE 14.—Diagrammatic representation of intrachromosomal exchange. If the exchange occurs within the newly synthesized chromatids or within the old ones there is loss of cistrons in the form of a ring. [Redrawn from Peterson and Laughnan (49).]

females of *bobbed* stocks and rRNA showed a considerable fluctuation. Values lower than that reported in the figure have been obtained as well as higher values. Sometimes the value was somewhat higher than 0.135 but always significantly lower than wild type. An appreciable number of non-*bobbed* females was unfortunately almost always present in these stocks. We propose that the mechanism responsible for the reversion of the *bobbed* stocks to wild type is unequal crossing-over. A classical scheme of this is represented in text-figure 12 which shows how crossovers can result in numerical differences. In this scheme all the chromosomal regions are paired.

THE NUMBER OF CISTRONS FOR rRNA AS AN EQUILIBRIUM VALUE

What we are essentially proposing is that the estimation of about 130 cistrons for rRNA per haploid wild-type genome is not a constant charac-

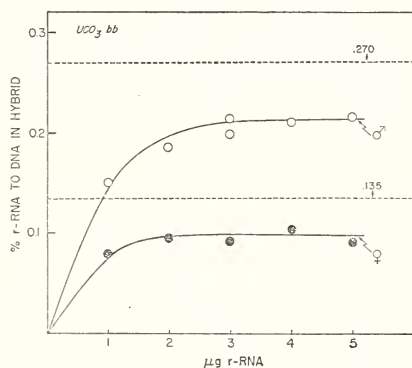
teristic for every individual of the population. We suggest rather that the number found in individuals will form a statistical distribution around a mean of 130. The following predictions emerge from these considerations.

1) *Bobbed* mutant alleles should appear in a wild-type population with a frequency per generation which should correspond to the probability of the occurrence of unequal crossover products which contain less than *one half* of a normal set of rRNA cistrons. We define a *bobbed* allele as one yielding the *bobbed* phenotype in the homozygous state.

2) In conditions in which crossing-over can occur the ♀ (4) and the ♂ (3) stock should progressively lose rRNA cistrons and reach a plateau at 0.27%.

3) *Bobbed* mutations should be obtainable out of females originally with 4 complete nucleolar organizers.

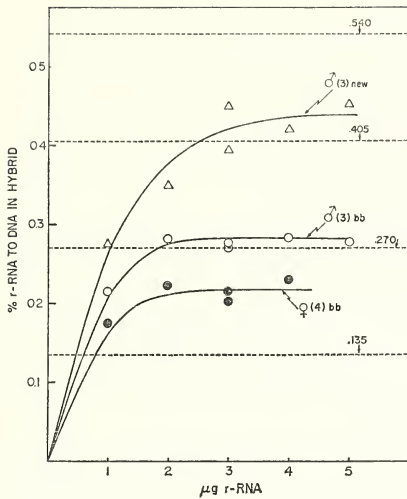
In conformity with the first prediction, a very high frequency of *bobbed* mutants in wild-type populations has been reported (50). We have also studied the presence of *bobbed* mutations in laboratory populations. The test consists in crossing wild-type females with *sc⁴-sc⁸* males and scoring for *bb* mutations among their daughters. While the major part of these F₁ females are wild type, a significant number of *bobbed* mutants has been found. Several lines have been kept and tested in hybridization experiments. The X chromosome of one of these (UCO 3 stock) has been made homozygous, and the relative saturation curves (text-fig. 15) demonstrate a clear deficiency for rDNA.



TEXT-FIGURE 15.—Saturation levels of DNA from a newly isolated *bobbed* stock with rRNA. The stock is UCO 3 *bb* and was obtained as described in the text.

Bobbed mutations are spontaneously selected against, at least in artificial populations. This is shown by the degeneration of the *bobbed* stocks. This fact would lead to a progressive increase in the number of ribosomal cistrons per individual unless the individuals with more than 0.27% rRNA are also selected against.

In agreement with the second and third predictions, spontaneous loss of rRNA cistrons occurs since we have been able to obtain *bb* mutants out of our female stocks which originally carried 4 NO regions. The level of saturation of the DNA from these flies only partially purified is shown in text-figure 16.



TEXT-FIGURE 16.—Saturation levels of DNA from various stocks carrying the $sc^{sIL}sc^{4R}$ chromosome. ♂ (3) bb and ♀ (4) bb once gave saturation plateaus, respectively, around 0.405 and 0.540% (text-fig. 2). The stock $sc^{sIL}sc^{4R}/sc^{sIL}sc^{4R}$ ♀; $sc^{sIL}sc^{4R}/Y$ ♂ was then kept as such for several generations. Later, males of this stock were crossed to tester *bobbed* females ($y w bb^{ds}$ stock). The $sc^{sIL}sc^{4R}$ X chromosome from females of this cross showing *bobbed* phenotype was then made homozygous [♀ (4) bb] or combined with a Y [♂ (3) bb]. At the time of the hybridization test only a fraction of the females showed *bobbed* phenotype, however.

As a comparison, a saturation level is presented from ♂ (3) maintained in the stock of the following sex chromosome constitution: ♂ $sc^{sIL}sc^{4R}/Y$; ♀ XX/Y . See text for discussion.

We might note here a fortunate circumstance concerning the analysis of the sc^s-sc^4 chromosome. In the original stock used, the sc^s-sc^4 chromosome was present only in the males of the stock, the females being of the $XX Y$ type. The frequency of crossing-over in *Drosophila* males is notoriously very low. The sc^s-sc^4 chromosome was made homozygous and tested immediately in hybridization experiments. If the chromosome had been maintained for a long time in females, the results would very likely have been entirely misleading.

These data favor the idea that unequal crossing-over is responsible for the recorded changes in the numbers of cistrons for rRNA. That this is so, however, remains to be shown. Whatever the molecular basis may be, the data show that when the machinery for ribosomal RNA formation is depleted or augmented it may spontaneously readjust to normal over the course of generations.

CONCLUSIONS

Experiments are described which were designed to illuminate the relation of ribosomal (rRNA) and transfer RNA (tRNA) species to the genome. The various conclusions derivable from the data obtained are briefly summarized.

Molecular hybridization experiments with labeled rRNA and DNA prepared from *Drosophila* stocks carrying different doses of the nucleolar organizer (NO) region lead to the following conclusions:

THE NUCLEOLUS

1) The wild-type genome saturates at 0.27% of the DNA, which indicates that for each of the two rRNA components approximately 130 complementary sites exist per haploid set.

2) The NO regions on the X and Y chromosome contribute equally to the proportion of DNA complementary to rRNA.

3) The proportions of the DNA found to be complementary to rRNA in the different stocks correspond to those predicted from their genetic constitutions and the assumption that all the DNA complements of rRNA are confined to the NO region.

4) By identifying the NO segment as the site of the required DNA templates, the data support the assertion that the nucleolus is the site of rRNA synthesis.

By means of competitive hybridization with tritium-labeled 28S rRNA and P³²-labeled 18S RNA, it was established that these two ribosomal components of *Drosophila melanogaster* are generated by their own distinct DNA templates.

By means of experiments similar to those employed for rRNA, analogous questions were answered with respect to tRNA. The saturation values obtained indicate that approximately 0.018% of the DNA of *D. melanogaster* is complementary to tRNA. This number leads to an approximately 15-fold redundancy for each of the approximately 60 tRNA species. Hybridization by use of DNA from the stocks carrying the various doses of the NO region established that tDNA cannot be detected in the region of the genome that has been shown to contain the complete cluster of the rDNA complements.

Chromosomal or nonchromosomal location of the rRNA cistrons was examined by means of saturation hybridization with DNA derived from tissues differing in the extent to which they are engaged in rRNA synthesis. These included sperm, erythrocytes, reticulocytes, embryo, kidney, liver of the chicken, etc. The saturation plateaus of the DNA from these various tissues were essentially identical within experimental error. The results are therefore consistent with chromosomal redundancy rather than parallel replication of extra copies.

Certain correlative properties of the *bobbed* mutation, located close to the NO, and the properties to be expected of stocks carrying deletions of ribosomal DNA segments led to the hypothesis that the *bobbed* region contains rDNA. This hypothesis was tested by examination of a number of *bobbed* mutants and it was found that their saturation plateaus are significantly lower than normal, often less than half of wild type. In addition, the hypothesis would predict that the length of the rDNA segment could be modified either up or down by means of unequal crossing-over. This in turn led to the prediction that females carrying an extra set of NO regions might suffer a loss in their rDNA and return to normal. This expectation was confirmed. Finally, it should be possible to actually derive *bobbed* females from such stocks and this also was confirmed.

The experiments reported here illustrate the potential usefulness of the fusion of classical genetic methodology and materials with the more recently developed techniques of molecular matching. The result generates possibilities of answering questions that were not previously amenable to experimental resolution.

RESUMEN

Los experimentos de hibridación molecular con ARN ribosómico marcado (ARN-r) y ADN de cepas de *Drosophila* portadoras de diferentes dosis del organizador nucleolar (NO) mostraron que el genomio de tipo salvaje satura al 0,27% del ADN indicando que, para cada uno de los dos componentes ARN-r, existen aproximadamente 130 sitios complementarios por juego haploide.

Las proporciones de ADN complementario del ARN ribosómico en las diferentes cepas corresponden a las predichas conforme a sus constituciones genéticas y a la suposición de que todos los complementos de ADN del ARN ribosómico están limitados a la región del organizador nucleolar.

La hibridación competitiva con ARN-r marcado con tritio y ARN 18S marcado con P^{32} estableció que estos dos componentes ribosómicos de *D. melanogaster* son generados por sus propios moldes distintivos.

Se respondieron a cuestiones análogas con respecto a la transferencia del ARN (ARN-t). Los valores de saturación obtenidos indican que aproximadamente 0,018% del ADN de *Drosophila melanogaster* es complementario del ARN-t. Este número conduce a una redundancia de aproximadamente 15 veces mayor para cada una de las aproximadamente 60 especies de ARN-t. La hibridación efectuada con ADN de cepas que portan diversas dosis de la región NO estableció que el ADN-t no puede ser detectado en esa región.

La localización cromosómica o no cromosómica de los cistrones del ARN-r fue examinada mediante hibridación por saturación con ADN obtenido de tejidos que difieren en la extensión en que están comprometidos en la síntesis del ARN-r. Estos incluyeron espermatozoides, eritrocitos, embriones, riñón e hígado de pollo. Los plateaus de saturación del ADN de estos diversos tejidos fueron esencialmente idénticos dentro del error experimental.

Ciertas propiedades de la mutación *bobbed* que está localizada cerca del organizador nucleolar condujeron a la hipótesis de que la región *bobbed* contiene ADN-r. Se examinó un número de mutantes *bobbed* y se halló que sus plateaus de saturación son significativamente más bajos que lo normal.

REFERENCES

- (1) CASPERSSON, T.: Cell growth and cell function. New York, Ed Norton, 1950.
- (2) BRACHET, J.: Biochemical Cytology. New York, Academic Press Inc., 1957.
- (3) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. Proc Nat Acad Sci USA 48: 2179-2186, 1962.
- (4) EISTRÖM, J.-E., GRAMPP, W., and SCHOR, N.: The intracellular distribution and heterogeneity of ribonucleic acid in starfish oocytes. J Biophys Biochem Cytol 11: 549-557, 1961.
- (5) BROWN, D. D., and GURDON, J. B.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. Proc Nat Acad Sci USA 51: 139-146, 1964.
- (6) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. Proc Nat Acad Sci USA 53: 737-745, 1965.

- (7) CHIPCHASE, M. I. H., and BIRNSTIEL, M. L.: On the nature of nucleolar RNA. *Proc Nat Acad Sci USA* 50: 1101-1107, 1963.
- (8) HALL, B. D., and SPIEGELMAN, S.: Sequence complementarity of T₂-DNA and T₂-specific RNA. *Proc Nat Acad Sci USA* 47: 137-146, 1961.
- (9) YANKOFSKY, S., and SPIEGELMAN, S.: The identification of the ribosomal RNA cistron by sequence complementarity. I. Specificity of complex formation. *Proc Nat Acad Sci USA* 48: 1069-1078, 1962.
- (10) McCONKEY, E. H., and HOPKINS, J. W.: The relationship of the nucleolus to the synthesis of ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 51: 1197-1204, 1964.
- (11) BIRNSTIEL, M. L., and CHIPCHASE, M. I. H.: The chemical and physical fractionation of nucleoli. *Fed Proc* 22: 473, 1963.
- (12) BIRNSTIEL, M. L., FLEISSNER, E., and BOREK, E.: Nucleolus: a center of RNA methylation. *Science* 142: 1577-1580, 1963.
- (13) SIRLIN, J. L., JACOB, J., and TANDLER, C. J.: Transfer of the methyl group of methionine to nucleolar ribonucleic acid. *Biochem J* 89: 447-452, 1963.
- (14) BIRNSTIEL, M. L., SIRLIN, J. L., and JACOB, J.: The nucleolus: a site of transfer ribonucleic acid synthesis. *Biochem J* 94: 10-11, 1965.
- (15) GERSHENSON, S. M.: Studies on the genetically inert region of the X-chromosome of *Drosophila melanogaster*. I. Behaviour of an X-chromosome deficient for a part of its inert region. *J Genetics* 28: 297-313, 1933.
- (16) GILLESPIE, D., and SPIEGELMAN, S.: A quantitative assay for DNA-RNA hybrids with DNA immobilized on a membrane. *J Molec Biol* 12: 829-842, 1965.
- (17) COOPER, K. W.: Cytogenetic analysis of major heterochromatic elements (especially Xh and Y) in *Drosophila melanogaster*, and the theory of "heterochromatin." *Chromosoma* 10: 535-588, 1959.
- (18) RUDKIN, G. T.: The structure and function of heterochromatin. *In Genetics Today. Proc. XI Int. Confr. Genetics. The Hague, The Netherlands, vol 2, 1963, pp 359-374.*
- (19) SWIFT, H.: Personal communication.
- (20) YANKOFSKY, S. A., and SPIEGELMAN, S.: Distinct cistrons for the two ribosomal RNA components. *Proc Nat Acad Sci USA* 49: 538-544, 1963.
- (21) ATTARDI, G., HUANG, P., and KABAT, S.: Recognition of ribosomal RNA sites in DNA. II. The HeLa cell system. *Proc Nat Acad Sci* 54: 185-193, 1965.
- (22) RITOSSA, F. M., and SPIEGELMAN, S.: Evidence for distinct cistrons for the two ribosomal RNA species of *D. melanogaster*. In preparation.
- (23) SCHERRER, K., and DARNELL, J. E.: Sedimentation characteristics of rapidly labelled RNA from HeLa cells. *Biochem Biophys Res Commun* 7: 486, 1962.
- (24) HAYASHI, M., and SPIEGELMAN, S.: The selective synthesis of informational RNA in bacteria. *Proc Nat Acad Sci USA* 47: 1564-1580, 1961.
- (25) GIACOMONI, D., and SPIEGELMAN, S.: Origin and biologic individuality of the genetic dictionary. *Science* 138: 1328-1331, 1962.
- (26) GOODMAN, H. M., and RICH, A.: Formation of DNA-soluble RNA hybrid and its relation to the origin, evolution and degeneracy of soluble RNA. *Proc Nat Acad Sci USA* 48: 2101-2109, 1962.
- (27) RITOSSA, F. M., ATWOOD, K. C., and SPIEGELMAN, S.: On the redundancy of DNA complementary to amino acid transfer RNA and its absence from the nucleolar organizer region of *Drosophila melanogaster*. In manuscript.
- (28) LEWIS, E. B.: Pseudoallelism and gene evolution. *Sympos Quant Biol* 16: 159-174, 1951.
- (29) STEPHENS, G. S.: "Homologous" genetic loci in *Gossypium*. *Symp Quant Biol* 16: 131-142, 1951.
- (30) VERMEULEN, C. W., and Atwood, K. C.: The proportion of DNA complementary to ribosomal RNA in *Drosophila melanogaster*. *Biochem Biophys Res Commun* 19: 221-226, 1965.

- (31) ATWOOD, K. C., and WHITE, J. L.: A hypothesis concerning the *Minutes* in *Drosophila melanogaster*. In manuscript.
- (32) WALLACE, H., and BIRNSTIEL, M. L.: Ribosomal cistrons and the nucleolar organizer. *Biochim Biophys Acta* 114: 296-310, 1966.
- (33) LIMA-DE-FARIA: Progress in tritium autoradiography. *Progr Biophys* 12: 282-317, 1962.
- (34) MILLER, O. L.: Extrachromosomal nucleolar DNA in amphibian oocytes. *J Cell Biol* 23: 60A, 1964.
- (35) PEACOCK, W. J.: Chromosome replication. *Nat Cancer Inst Monogr* 18: 101-131, 1965.
- (36) RITOSSA, F. M., BLEYMAN, M., and SPIEGELMAN, S.: Chromosomal redundancy of DNA complementary to ribosomal RNA. In manuscript.
- (37) GREEN, M. M.: Pseudoallelism and the gene concept. *Amer Natur* 89: 65-71, 1955.
- (38) STEFFENSEN, D.: A comparative view of the chromosome. *Brookhaven Sympos Biol* 12: 103-124, 1959.
- (39) TROSKO, J. E., and WOLFF, S.: Strandedness of *Vicia faba* chromosomes as revealed by enzyme digestion studies. *J Cell Biol* 26: 125-136, 1965.
- (40) YANKOFSKY, S. A., and SPIEGELMAN, S.: The identification of the ribosomal RNA cistron by sequence complementarity. II. Saturation of and competitive interaction at the RNA cistron. *Proc Nat Acad Sci USA* 48: 1466-1472, 1962.
- (41) MCCLINTOCK, B.: The relationship of a particular chromosomal element to the development of the nucleoli in *Zea Mays*. *Z Zellforsch* 21: 294-328, 1934.
- (42) KRIVSHENKO, J.: The divisibility of the nucleolus organizer and the structure of the nucleolus in *Drosophila busckii*. *Genetics* 44: 520-521, 1959.
- (43) BEERMAN, W.: Der Nukleolus als Lebenswichtiger Bestandteil des Zellkernes. *Chromosoma* 11: 263-296, 1960.
- (44) STURTEVANT, A. H.: The effects of unequal crossing over at the Bar locus in *Drosophila*. *Genetics* 10: 117-147, 1925.
- (45) JUDD, B. H.: Formation of duplication—deficiency products by asymmetrical exchange within a complex locus of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 47: 545-550, 1961.
- (46) STERN, C.: Ein genetischer und zytologischer Beweis für Ververbung in Y-chromosome von *Drosophila melanogaster*. *Z Indukt Abstammungsvererbungst* 44: 187-231, 1927.
- (47) KIKKAVA, H.: Studies on the genetics and cytology of *Drosophila ananassae*. *Genetics* 20: 458-516, 1938.
- (48) STERN, C.: Über die additive Wirkung multipler Allele. *Biologisches Zentralblatt* 49: 261-290, 1929.
- (49) PETERSON, H. M., and LAUGHNAN, J. R.: Intrachromosomal exchange at the Bar locus in *Drosophila*. *Proc Nat Acad Sci USA* 50: 126-133, 1963.
- (50) SPENCER, W. P.: Iso-alleles at the bobbed locus in *Drosophila hydei* populations. *Genetics* 29: 520-536, 1944.

DISCUSSION

Waddington: I was very interested in your point that *Drosophila* females having three or four nucleolar organizers generally revert toward the normal in conditions where crossing-over can occur. I can see that they possibly could revert, but what is the reason that they actually do revert?

Ritossa: We are proposing that not every individual of the population carries exactly the 130 ribosomal cistrons that we find per haploid genome of a wild population, but rather that individuals of the population possess haploid genomes that

give a mean of 130 cistrons. That is, we think there are a certain number of individuals containing less than 130 cistrons and a part of the population which contains more. We know there are individuals with less than 130 cistrons because we have been able to isolate large numbers of *bobbed* mutants out of wild populations. The *bobbed* stocks revert to wild type, which indicates that the *bobbed* "mutation" is selected against. This would imply that in a population there is a constant loss of individuals with a low number of ribosomal cistrons. This would cause a progressive accumulation of ribosomal cistrons per chromosome within the population. If the population is to remain in equilibrium, there must also be selection against those individuals with more than the mean value.

Birnstiel: When we prepare rRNA from *Xenopus* we notice that it is extremely difficult to free it completely from messenger RNA, and this is also true for 4S RNA. Could you therefore assure us that when you measure RNA hybrids with soluble RNA you are actually measuring only tRNA-DNA hybrids?

Ritossa: In the case of rRNA the purity is easily tested because if we plot number of nucleolar organizer regions versus saturation levels, the extrapolation passes through the origin. The only thing I can say is that with the DNA to RNA ratios we used, one generally cannot reach a plateau with messenger, while with tRNA we obtained a very nice plateau.

Sirlin: I am not sure but that I detect a flaw in your argument about the distribution of the DNA cistrons, based on your measurements of the relative amounts of hybridization in different tissues that are or are not synthesizing rRNA. Were you assuming, for instance, that the erythrocyte doesn't have DNA like this?

Ritossa: No. I said we did the experiment with the chicken erythrocytes to determine if there was a considerable amount of metabolic DNA. We found the same saturation level in all these DNA preparations, and we conclude that there are 100 cistrons in each of the chicken tissues analyzed.

Sirlin: Were you expecting those tissues not synthesizing rRNA to have less hybridizing DNA on this scheme for the DNA?

Ritossa: Yes, this was the current hypothesis.

Sirlin: But you find the same amount?

Ritossa: Yes.

Sirlin: The point is that you get the same hybridization ratios whether the DNA is being utilized in the cell or not, because when you denature it, it will form hybrids.

Ritossa: You are right. This is a possibility. This suggestion, however, is contrary to another expectation for metabolic DNA: instability.

THE NUCLEOLUS AND RIBOSOME BIOGENESIS

An undercurrent, which has frequently surfaced throughout these proceedings, is the belief or conviction that a primary function of the nucleolus, if not its sole function, is ribosome production. This problem is specifically examined in the final group of contributions to the Symposium where the interrelations between the RNA of nucleus, nucleolar fractions, and ribosomes are examined with the entire armamentarium of molecular biology. This session, which was chaired by Dr. C. Basilio and Dr. H. W. Lewis, is completed by a panel discussion among six of the Symposium contributors: Dr. M. Birnstiel, Dr. D. Brown, Dr. J. Gall, Dr. S. Penman, Dr. R. P. Perry, as moderator, and Dr. W. S. Vincent. This discussion serves to integrate many observations which had been presented throughout the preceding reports, as well as to raise in a pointed manner some of the still unanswered questions regarding the nucleolus.

Unfortunately, Dr. Georgiev was unable to present his paper in person, so that no discussion could be included.

Nuclear RNA of the Salamander Oocyte^{1, 2}

JOSEPH G. GALL,³ *Biology Department, Yale University,
New Haven, Connecticut*

SUMMARY

The mature amphibian oocyte contains 4 μ g or more RNA, mostly as 28S and 18S ribosomal RNA in the cytoplasm. A study has been made of the nuclear RNA in immature salamander oocytes with uridine-H³ as precursor. The giant nuclei were isolated manually from unfixed cells, the RNA was extracted by a phenol-detergent method, and sucrose gradient centrifugation was carried out. The earliest nuclear incorporation was in a 40S fraction; this was followed by the appearance of a 30S peak. Smaller 18S and 4S peaks were present in all gradients. Even after prolonged incorporation (8 days) the most prominent peaks remained at the 40S and 30S regions.

The earliest cytoplasmic incorporation was in the 18S ribosomal fraction, followed later by the 28S. These findings suggest that the ribosomal precursor RNA remains in the 40S and/or 30S form until the time it passes to the cytoplasm as definitive ribosomal RNA. They also indicate that only a small portion, if any, of the newly formed ribosomes end up in the nuclei of these cells. Some intranuclear localization of fractions was made by separation of cytological components (chromosomes, nucleoli, sap) before extraction of RNA.—*Nat Cancer Inst Monogr* 23: 475–488, 1966.

OOCYTES of most animals contain relatively large amounts of cytoplasmic RNA demonstrable by standard cytochemical methods (1, 2) and by biochemical fractionation (3–6). Individual mature oocytes of the toad *Xenopus laevis* contain up to 4 μ g of RNA (4, 5), and similar or higher values have been reported in the salamander, *Triturus* (7). Biochemical fractionation shows that most of this RNA is of the ribosomal type, with somewhat smaller amounts of material sedimenting at 4S. Since the oocyte shows a remarkable increase in size and RNA content during oogenesis, it is not surprising that the incorporation of RNA precursors is readily demonstrable. Earlier autoradiographic studies (8, 9) have been complemented by more recent biochemical analyses (3–6). The autoradio-

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported by Public Health Service research grant GM-12427 from the National Institute of General Medical Sciences.

³ The technical assistance of Mrs. Diane Dexter is gratefully acknowledged.

graphic evidence suggested that the earliest incorporation is nuclear. The biochemical analyses, based on whole oocytes, indicate that the labeled precursors end up primarily in ribosomal RNA and 4S RNA.

Oocyte nuclei are particularly suited for studies of RNA synthesis because of their large size and the ease with which they can be isolated by hand from fresh oocytes. In addition, extensive studies on the giant chromosomes and multiple nucleoli of the oocyte provide an opportunity for correlating biochemical and cytochemical findings. For these reasons the present studies were carried out to define the nuclear RNA fractions in terms of sedimentation rate, labeling patterns, and cytological localization.

MATERIALS AND METHODS

Preparation of unlabeled oocyte RNA.—In most sedimentation experiments, unlabeled oocyte RNA was used as a velocity marker. Fresh ovaries of the newt, *Triturus viridescens*, or the axolotl, *Amblystoma mexicanum*, were homogenized in ice-cold 0.1 M sodium acetate, pH 5.0, containing 0.5% sodium dodecyl sulfate (SDS). An equal volume of water-saturated phenol was then added. After further homogenization the aqueous layer was removed by centrifugation. Magnesium chloride was added to a final concentration of 0.01 M, and the resulting white precipitate centrifuged. The supernatant contains RNA which is precipitated by adding two volumes of cold ethanol. Details of the procedure are as described by Brown and Littna (4, 5). Base composition of nonradioactive RNA was determined by electrophoresis on cellulose acetate strips after alkaline hydrolysis.

Preparation of labeled nuclear and cytoplasmic RNA.—In some cases, oocytes were labeled by injecting uridine- H^3 into the coelomic cavity of living newts. Much higher levels of incorporation are obtained if one dissects out the ovary and places it in a radioactive solution. In a typical experiment one ovary of *T. viridescens* was placed in 1.0 ml culture medium containing 100 μ c uridine- H^3 of specific activity 1–5 c/mmole. A satisfactory culture medium consisted of 0.6 ml commercial medium 199, 0.1 ml calf serum, and 0.3 ml water. Penicillin G (0.06 mg/ml) and streptomycin (0.1 mg/ml) were added to retard the growth of microorganisms. In this medium oocytes will continue to incorporate uridine for several days, and the morphology of lampbrush chromosomes isolated from the nuclei is normal in many of the cells. Nuclei were individually isolated from unfixed oocytes by procedures previously described (10, 11). In most experiments, the nuclei were transferred from the buffered saline in which they were isolated to 0.1 M sodium acetate, pH 5.0, containing 4 μ g/ml polyvinyl sulfate (PVS). In this medium they are “fixed,” that is, they become hard and white, and are consequently easier to handle. If SDS is added to a final concentration of 0.5% the nuclei go into solution. Such detergent-treated material may be layered directly on sucrose gra-

dients for analysis of the released RNA. In most instances, however, the RNA was further purified by extracting in the cold for 10–30 minutes with an equal volume of phenol to remove protein. Unlabeled oocyte RNA was added (to the aqueous layer) to act as carrier, and the RNA was precipitated with 2 volumes of cold ethanol.

The isolation of oocyte cytoplasm was facilitated by first fixing bits of ovary in ice-cold 70% ethanol. The follicular membranes were removed from each oocyte by means of forceps. The oocyte was then opened and the nucleus removed. The cytoplasm, which was usually broken into several pieces, was next transferred to 0.1 M sodium acetate, pH 5.0, and the RNA extracted by the detergent-phenol method already described. If radioactive RNA from a few oocytes is to be examined, unlabeled RNA may be added as carrier. If 100 or more oocytes are used, they will contain enough RNA for direct isolation.

After the final ethanol precipitation, the RNA was taken up in 1.0 ml 0.01 M sodium acetate, pH 5.0, containing 1 μ g/ml PVS, and layered on top of a 30 ml sucrose gradient. Most of our work has involved linear 10–30% sucrose gradients made up in 0.01 M sodium acetate, pH 5.0, containing 10^{-4} M EDTA, as recommended by Brown (4, 5). RNA sediments more slowly in this medium than in one of higher ionic strength, but the radioactivity bands appear to be sharper. Centrifugation was carried out at 10–15° C for 15–18 hours at 25,000 rpm in the Spinco SW-25 swinging bucket rotor. At the end of the run approximately thirty 1 ml fractions were obtained by puncturing the bottom of the tube. The optical density of each fraction was measured at 260 $m\mu$ to give the distribution of the carrier RNA. Each fraction was then diluted with 10 ml Bray's solution (12), and radioactivity measured in a scintillation counter.

Sedimentation coefficients were measured by band centrifugation in the analytical ultracentrifuge as described by Vinograd, Bruner, Kent, and Weigle (13). Twenty μ l of RNA solution were layered onto 0.5 M KCl buffered either to pH 7.0 with 0.08 M phosphate or to pH 5.0 with 0.01 M acetate. The RNA bands were photographed with ultraviolet optics, and densitometer tracings were made of the photographs.

RESULTS

Sedimentation Pattern of Unlabeled Ovarian RNA

The RNA extracted by the SDS-phenol method from salamander ovaries sedimented in three bands in sucrose gradients. Similar behavior has been previously reported for the ovarian RNA of the toad, *Xenopus* (4, 5). The bands corresponded to material conventionally referred to as 28S and 18S ribosomal RNA and 4S transfer RNA. Sedimentation coefficients were determined in the analytical ultracentrifuge in three solvents, and the values are summarized in table 1. It will be noted that the sedimentation coefficients are dependent on salt concentration. In 0.5 M KCl,

TABLE 1.—Sedimentation coefficients of ($s_{20,w}$) ovarian RNA from the newt, *Triturus viridescens*

Conditions	Class of RNA			Number of determinations
	"28S"	"18S"	"4S"	
Boundary 0.01 M Sodium acetate 10 ⁻⁴ M EDTA pH 5	22.3	13.7	3.6	1
Band (Vinograd) 0.5 M KCl 0.08 M PO ₄ pH 7	29.8	18.5	5.3	5
Band (Vinograd) 0.5 M KCl 0.01 M Sodium acetate 10 ⁻⁴ M EDTA pH 5	31.1	19.5	4.5	2

pH 5.0 or 7.0, the $s_{20,w}$, was above the conventional value; in 0.01 M Na acetate pH 5.0, it was below. The relative values for the two ribosomal peaks were the same in all cases. For convenience in describing the sucrose gradient experiments, the peaks will be referred to by their conventional values of 28S, 18S, and 4S.

Data on the base composition of these three fractions are given in table 2. All are of the high G + C type, as previously reported for *Xenopus* (4) and other organisms.

TABLE 2.—Base composition of RNA fractions from oocytes of the newt, *Triturus viridescens*. Molar proportions determined by electrophoresis on cellulose acetate strips

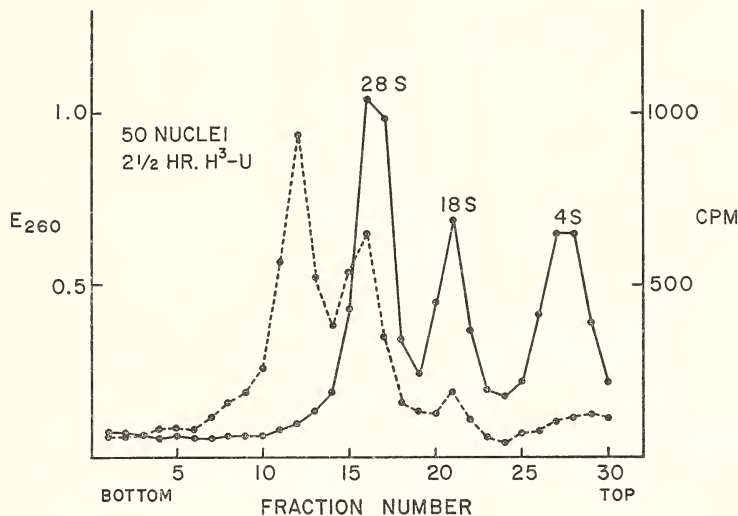
Type of RNA	A*	G*	C*	U*	G + C	n*
28S.....	21.8	35.0	25.9	17.3	60.9	7
18S.....	24.9	31.1	24.4	19.6	55.5	6
4S.....	22.3	34.0	25.9	17.8	59.9	3

*A, G, C, U = adenine, guanine, cytosine, uracil; n = number of analyses.

Incorporation of Uridine-H³ Into Nuclear RNA

When oocytes were kept in uridine-H³, either in Ringer's solution for short-term experiments or in culture medium for experiments lasting longer than 12 hours, the nuclear RNA continued to label at an approximately linear rate. At the same time the cytoplasmic RNA became progressively more heavily labeled. In a typical experiment in which oocytes were incubated 12 hours in a medium containing uridine-H³ at 100 μ c/ml,

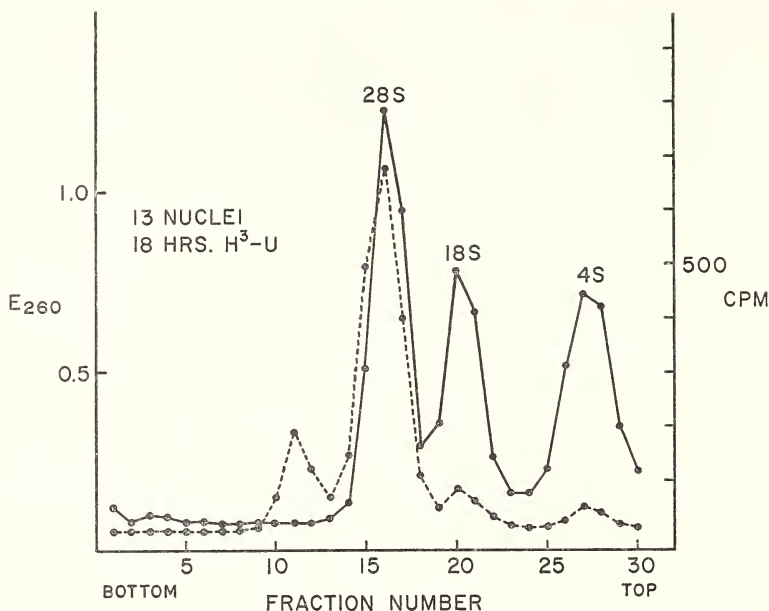
individual nuclei might show 100–200 cpm in RNA. In longer experiments lasting 3 or 4 days, individual nuclei contained 1000–2000 cpm in RNA. Since the nucleus contained only 0.02–0.03 μg RNA (14, 15), the latter values corresponded to specific activities of about $5\text{--}10 \times 10^4$ cpm per μg RNA.



TEXT-FIGURE 1.—Sedimentation pattern of radioactivity in oocyte nuclear RNA after incubation of salamander ovary for 2½ hours in uridine-H³. In this and subsequent text-figures the *dotted line* refers to radioactivity. The *solid line* represents the optical density of nonradioactive whole cell RNA added as a velocity marker. Experimental details are given under Materials and Methods. E₂₆₀ = optical extinction at 260 m μ . CPM = counts per minute by scintillation, not corrected for counting efficiency.

The sedimentation pattern of the nuclear radioactivity in short-term experiments is illustrated in text-figure 1. In this example, nuclei isolated after 2½ hours of continuous incorporation showed two major and two minor peaks. Both major peaks ran ahead of the 28S marker, although the slower of the two was displaced only one fraction toward the bottom of the tube. This slight displacement, which in a single run might be considered fortuitous, is completely reproducible. If one assigns a value of 28.0S to the heavy ribosomal peak and further assumes that S value varies linearly with distance traveled in the sucrose gradient (16), then the radioactivity peaks fall at values of 30.0S and 40.1S (averages of 24 determinations). As previously mentioned, in 0.01 M acetate-EDTA the true values were less than this, but for convenience these peaks will be referred to by their nominal values. The two minor peaks in the radioactivity profile are coincident with the 18S and 4S marker peaks.

Longer incubation in uridine-H³ led to patterns with the same four peaks but with altered relative heights (text-fig. 2). The 30S peak

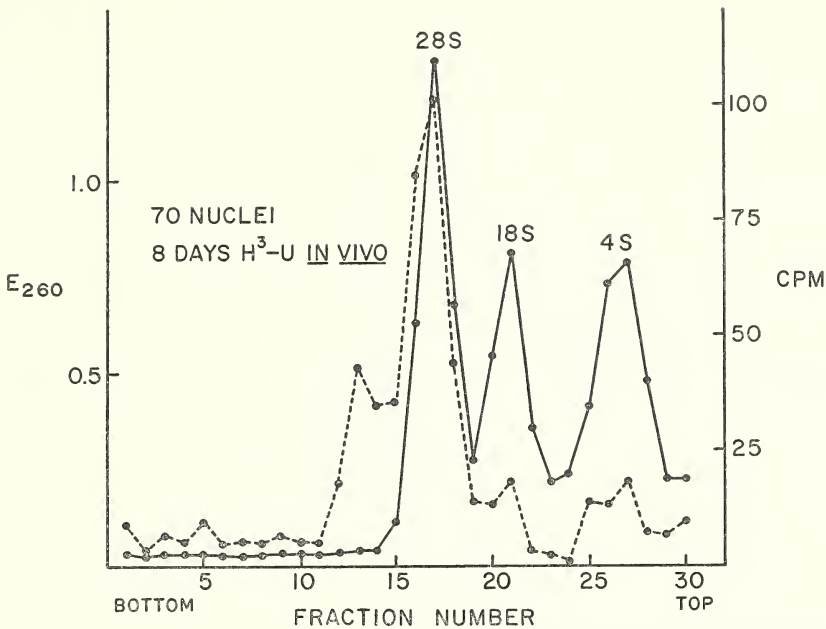


TEXT-FIGURE 2.—Sedimentation pattern of radioactivity in oocyte nuclear RNA after incubation of salamander ovary for 18 hours in uridine- H^3 . The major radioactivity peak consistently runs slightly ahead of the nonradioactive 28S velocity marker.

came to dominate the pattern, the 40S, 18S, and 4S material contributing smaller peaks in the order named. All incubations lasting more than about 12 hours gave similar results. In one experiment an animal was given 2 intraperitoneal injections of uridine- H^3 spaced 2 days apart, and oocyte nuclei were isolated 8 days after the first injection. The pattern of nuclear radioactivity was essentially identical to that seen in shorter *in vitro* experiments (text-fig. 3).

Incorporation of Uridine- H^3 Into Cytoplasmic RNA

An advantage of oocytes is the ease with which nuclei can be isolated manually for study. The enucleated oocytes also can be examined for information concerning the cytoplasmic RNA. Defolliculated, enucleated oocytes were collected from ovaries incubated in uridine- H^3 for various times. In all cases three radioactivity peaks were found, coincident with the 28S, 18S, and 4S marker peaks. In shorter experiments, the 18S peak predominated (text-fig. 4), and in some cases there was almost no 28S radioactivity. With longer incubation periods, the 28S peak increased in relative height until after 12–18 hours of incubation the 28S and 18S radioactivity peaks had the same relative heights as in the optical density pattern. The 4S peak was not as prominent in the radioactivity profile as in the nonradioactive marker. The marker was isolated from whole

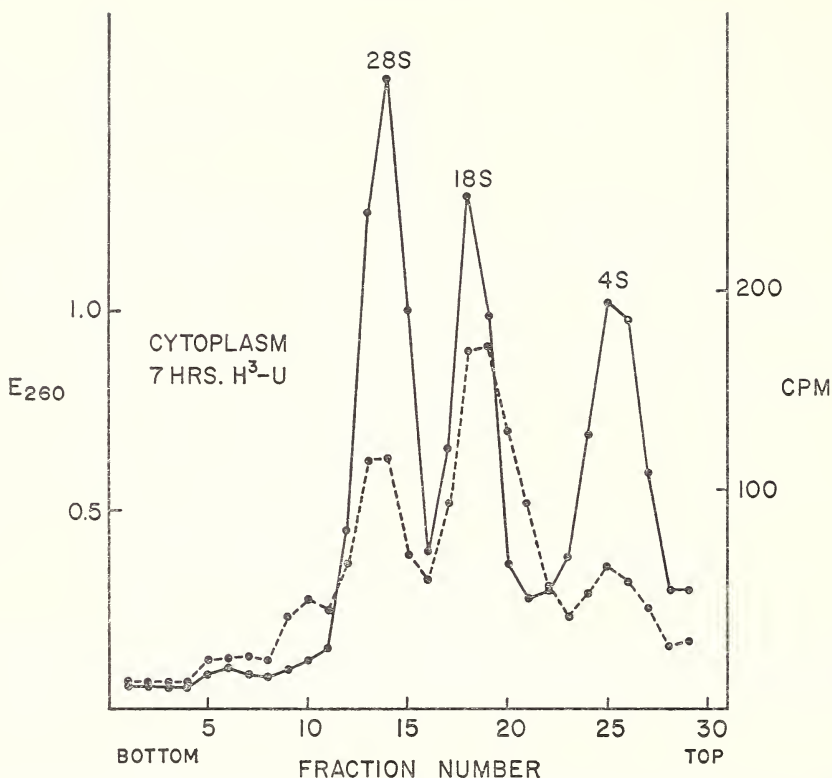


TEXT-FIGURE 3.—Sedimentation pattern of radioactivity in oocyte nuclear RNA 8 days after injection of uridine- H^3 into a salamander.

ovaries, whereas the radioactivity pattern came only from the cytoplasm of large oocytes. The origin of the 4S material in the marker is uncertain, though it may come primarily from the smaller oocytes or the follicle cells.

Optical Density Profile of Nuclear RNA

A single oocyte nucleus of *Triturus* contains about 0.02–0.03 μg RNA at the stage used in these studies (14, 15). Since one can easily collect 100 nuclei manually, it is possible to work with about 2–3 μg of nuclear RNA. With this amount one can examine the optical density profile by band centrifugation in the analytical ultracentrifuge. DNA is not a source of confusion in such an experiment since the RNA/DNA ratio is at least 20:1 in these nuclei (14, 17). In the tracer experiments described above, the nuclear RNA was isolated with the help of added nonradioactive carrier. Several attempts to purify the nuclear RNA without carrier led to degradation (failure of the ultraviolet-absorbing material to migrate in the ultracentrifuge). As an alternative we simply dissolved isolated nuclei in 20 μl of 1% SDS and layered the mixture directly onto 0.5 M KCl. Prior experiments with sucrose gradients showed that RNA was separated from protein by SDS alone. Two ultraviolet-absorbing bands were seen: a major band sedimenting with a sedimentation coefficient, $s_{20, w} = 3.3$, which presumably consisted largely of protein; and a less prominent band sedimenting with $s_{20, w} = 35.3$. The latter is tentatively identi-



TEXT-FIGURE 4.—Sedimentation pattern of radioactivity in oocyte cytoplasmic RNA after incubation of salamander ovary for 7 hours in uridine- H^3 . After shorter incubation the 28S radioactivity may be entirely missing. After longer periods the patterns of radioactivity and optical density in the 28S and 18S regions are similar.

fied as the bulk of the nuclear RNA. The heavy ribosomal peak run under identical conditions had an $s_{20, w} = 31.1$. Thus ultraviolet-absorbing material from the nucleus runs slightly faster than the heavy ribosomal peak, and therefore probably corresponds to the 30S radioactivity seen in the sucrose gradients.

DISCUSSION

Since the bulk of the RNA produced by the oocyte during its long period of growth is ribosomal, probably the radioactivity observed in the nucleus is largely ascribable to ribosomal precursors. Earlier studies have shown that ribosomal precursors occur as two components with sedimentation values of about 35S and 45S, presumably corresponding to our 30S and 40S fractions (18-23). According to the schemes discussed by Perry (21-23), Girard, Penman, and Darnell (19), and others, the earliest ribosomal precursor is the 45S material. This is converted partially or wholly into the 35S. Perry suggests that the 18S ribosomal fraction is

formed at the time the 45S is converted to 35S, and that the 28S ribosomal material is derived directly from the 35S. Girard *et al.* (19, 20), and Perry (23), demonstrated that the 18S ribosomal fraction labels faster than the 28S in cultures of HeLa cells and L cells, and the present results show the same situation in an entirely different system.

The nuclear radioactivity patterns never show more than a small peak at 18S. Furthermore the 30S peak remains sharp and consistently heavier than the 28S marker even after prolonged incubation. These results permit two conclusions about the oocyte system. First, the conversion of the ribosomal precursors into 18S and 28S ribosomal fractions must occur more or less concomitantly with their migration into the cytoplasm. The small 18S peak in the nuclear material could represent ribosomal RNA which has not yet been transported to the cytoplasm. Whether a small amount of 28S material exists in the nucleus is not certain, since it would be difficult to distinguish in the presence of the higher 30S peak. Second, there can be only slight accumulation of new (mature) ribosomes in these nuclei at the stages being analyzed. This follows from the failure to find much 18S or 28S radioactivity in the nuclei even after several days of incubation in uridine- H^3 . It should also be noted that the low level of these peaks in the nuclear material affords evidence that the nuclei are obtained free of contaminating cytoplasm. After several days of incubation in uridine- H^3 the total amount of label in the cytoplasmic ribosomes is several times that in the nucleus.

Although the labeling experiments indicate that few if any new ribosomes (containing 18S and 28S RNA) are accumulating in the nucleus, they do not exclude the possibility that the nucleus contains a ribosomal population acquired during the earlier stages of oogenesis. For this reason it was particularly important to obtain the optical density profile of the nuclear RNA. So far only two successful runs in the analytical ultracentrifuge have been obtained, but they agree in showing a single prominent peak heavier than the typical 28S ribosomal RNA. It will be necessary to analyze larger quantities of material in order to detect minor peaks. It is possible that the complete optical density profile will resemble the radioactivity profiles obtained after long incubation (text-fig. 2); this would imply that the various types of nuclear RNA come into equilibrium with added tracer after about 12 hours' incubation.

The experiments so far discussed do not permit cytological localization of the several categories of nuclear RNA. Some inferences may be drawn by combining data from other sources. In *Triturus* oocytes of about 1.0 mm diameter the nucleus contains 20,000–30,000 pg of RNA (14, 15). Cytologically this is divisible into chromosomal, nucleolar, and sap (nucleoplasmic) RNA. Analyses performed by Edström and Gall (17) indicate that the chromosomes contain about 1000 pg, and the nucleoli together account for another 2500 pg. Thus the bulk of the nuclear RNA must be ascribed to the sap.⁴ If the 30S peak seen in both the radioactivity and

⁴ The value for sap RNA (4000–6000 pg) reported by Edström and Gall (17) is too low. It was obtained from preparations containing only part of the sap.

optical density profiles represents the major RNA component of the nucleus, then it must be largely derived from the nuclear sap.

The experiments of Perry (21-23) strongly suggest that the 45S ribosomal precursor in HeLa cells is localized in the nucleoli. In several experiments we have attempted to obtain gradients from radioactive nucleoli without contamination from chromosomal and sap material. So far the physical separation of components has not been good, but in several cases some enrichment of 40S material has been found. It seems likely, therefore, that some of the 40S material is found in the nucleoli. Attempts have also been made to separate chromosomes from sap and nucleoli. In the few partially successful cases, the RNA from radioactive chromosome preparations has not formed distinct peaks, but has sedimented throughout the range from about 40S and smaller. It is uncertain, however, that proper conditions for isolating undegraded chromosomal RNA have been found.

Insofar as they can be interpreted in cytological terms the experiments reinforce the now well-known relationship between the nucleoli and the production of ribosomal RNA precursors (3, 21). In addition, they suggest that the 30S RNA, which represents the bulk of the nuclear RNA in these cells, is located in the nuclear sap. An earlier study by Edström and Gall (17) showed that the nucleolar RNA of *Triturus* oocytes is similar in base composition to the bulk cytoplasmic RNA (primarily ribosomal). By contrast, the chromosomal RNA differs considerably from either and more closely resembles the DNA. Analyses of nuclear sap RNA gave variable results with a tendency toward high uracil values. The base ratio results were interpreted to show a close relationship between nucleolar and ribosomal RNA. If the sap RNA represents ribosomal precursor as well, we should expect its base composition to resemble that of the 28S ribosomal fraction. Unfortunately, the base analyses were too variable to permit any conclusions.

A few words should be said about the chromosomal RNA synthesis, which has figured prominently in earlier autoradiographic studies (8, 9, 24). Autoradiographic studies of the giant lampbrush chromosomes are usually made on material manually isolated from unfixed nuclei and prepared in a fashion to avoid precipitated nucleoplasm (11). This not only gives a better morphological picture but also excludes the heavy radioactivity of the sap, which otherwise obscures the autoradiograph of the chromosomes. In terms of total nuclear RNA and radioactivity the chromosomes contribute only a minor fraction. There is, therefore, no necessary reason to suggest that the lampbrush chromosomes are actively engaged in ribosomal RNA synthesis. On the contrary, it seems more reasonable to suppose that the chromosomes are involved in producing RNAs of heterogeneous nature. It is now known that the nucleoli of the oocyte, which are physically separate from the chromosomes, contain DNA (14, 25, 26). It is probable, therefore, that they are the original sites of ribosomal RNA synthesis.

CONCLUSIONS

The following scheme is consistent with most of the findings on oocyte RNA, although it is by no means exclusive or complete. The original ribosomal precursor is made in the nucleoli as material sedimenting at 40S. This is converted into an 18S fraction which goes quickly to the cytoplasm, and a 30S fraction which accumulates in the nuclear sap. The 30S fraction is more slowly converted into the definitive 28S ribosomal RNA, which passes to the cytoplasm as soon as it is produced. There is little or no return of definitive 18S and 28S ribosomal RNA from the cytoplasm to nucleus. This formulation borrows heavily from the earlier discussions of Perry (21-23), Girard *et al.* (19, 20), and others. The major weaknesses of the scheme, as applied to the oocytes, are the reliance on the temporal sequence of labeling and the scanty information on base ratios or other chemical data linking the various fractions.

RESUMEN

El ovocito maduro de Anfibio contiene 4 μ g o más de ARN, la mayor parte como ARN ribosómico 28S y 18S en el citoplasma. Se ha hecho un estudio del ARN nuclear en ovocitos inmaduros de salamandra empleando uridina H^3 como precursor. Los núcleos gigantes se aislaron en forma manual de células no fijadas, el ARN se extrajo por el método del detergente-fenol, y la centrifugación se llevó a cabo en gradiente de sucrosa. La incorporación nuclear más temprana es en la fracción 40S siendo seguida ésta por la aparición del pico 30S. Los picos menores 18S y 4S están presentes en todos los gradientes. Aun después de una prolongada incorporación (8 días) los picos más prominentes persisten en las regiones 40S y 30S. La incorporación citoplásmica más precoz es en la fracción ribosómica 18S seguida después por la 28S. Estos hallazgos sugieren que el ARN precursor ribosómico permanece en la forma 40S y/o 30S hasta el momento en que pasa el citoplasma como ARN ribosómico definitivo. Ellos también indican que solo una pequeña porción, si existe alguna, de los ribosomas recién formados termina en los núcleos de estas células. Se ha realizado cierta localización intranuclear de las fracciones mediante la separación de los componentes citológicos (cromosomas, nucleolos, jugo) antes de la extracción del ARN.

REFERENCES

- (1) BRACHET, J.: La localisation des acides pentosenucleiques dans les tissus animaux et les oeufs d'amphibiens en voie de développement. Arch Biol (Liege) 53: 207-257, 1942.
- (2) CASPERSSON, T., and SCHULTZ, J.: Ribonucleic acids in both nucleus and cytoplasm, and the function of the nucleolus. Proc Nat Acad Sci USA 26: 507-515, 1940.
- (3) BROWN, D., and GURDON, J.: Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. Proc Nat Acad Sci USA 51: 139-146, 1964.
- (4) BROWN, D. D., and LITNA, E.: RNA synthesis during the development of *Xenopus laevis*, the South African clawed toad. J Molec Biol 8: 669-687, 1964.

- (5) BROWN, D. D., and LITTE, E.: Variations in the synthesis of stable RNA's during oogenesis and development of *Xenopus laevis*. *J Molec Biol* 8: 688-695, 1964.
- (6) DAVIDSON, E., ALLFREY, V., and MIRSKY, A.: On the RNA synthesized during the lampbrush phase of amphibian oogenesis. *Proc Nat Acad Sci USA* 52: 501-508, 1964.
- (7) OSAWA, S., and HAYASHI, Y.: Ribonucleic acid and protein in growing oocytes of *Triturus pyrrhogaster*. *Science* 118: 84-86, 1953.
- (8) FICQ, A.: L'étude autoradiographique de métabolisme des protéines et des acides nucléiques au cours de l'oogenèse chez les Batraciens. *Exp Cell Res* 9: 286-293, 1955.
- (9) GALL, J. G., and CALLAN, H. G.: Uridine- H^3 incorporation in lampbrush chromosomes. *Proc Nat Acad Sci USA* 48: 562-570, 1962.
- (10) CALLAN, H. G., and LLOYD, L.: Lampbrush chromosomes of crested newts, *Triturus cristatus* (Laurenti). *Phil Trans Roy Soc London B* 243: 135-219, 1960.
- (11) GALL, J. G.: Techniques for the Study of Lampbrush Chromosomes. In *Methods in Cell Physiology*. New York, Academic Press, vol II, 1966, pp 37-60.
- (12) BRAY, G. A.: A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal Biochem* 1: 279-285, 1960.
- (13) VINOGRAD, J., BRUNER, R., KENT, R., and WEIGLE, J.: Band-centrifugation of macromolecules and viruses in self-generating density gradients. *Proc Nat Acad Sci USA* 49: 902-910, 1963.
- (14) IZAWA, M., ALLFREY, V., and MIRSKY, A.: Composition of the nucleus and chromosomes in the lampbrush stage of the newt oocyte. *Proc Nat Acad Sci USA* 50: 811-817, 1963.
- (15) EDSTRÖM, J.-E., and GALL, J. G.: Unpublished observations.
- (16) MARTIN, R. G., and AMES, B. N.: A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J Biol Chem* 236: 1372-1379, 1961.
- (17) EDSTRÖM, J.-E., and GALL, J. G.: The base composition of ribonucleic acid in lampbrush chromosomes, nucleoli, nuclear sap, and cytoplasm of *Triturus* oocytes. *J Cell Biol* 19: 279-284, 1963.
- (18) SCHERRER, K., LATHAM, H., and DARNELL, J.: Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 49: 240-248, 1963.
- (19) GIRARD, M., PENMAN, S., and DARNELL, J.: The effect of actinomycin on ribosome formation in HeLa cells. *Proc Nat Acad Sci USA* 51: 205-211, 1964.
- (20) GIRARD, M., LATHAM, H., PENMAN, S., and DARNELL, J.: Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm. *J Molec Biol* 11: 187-201, 1965.
- (21) PERRY, R.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (22) ———: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat Cancer Inst Monogr* 14: 73-89, 1964.
- (23) ———: The nucleolus and the synthesis of ribosomes. *Nat Cancer Inst Monogr* 18: 325-340, 1965.
- (24) IZAWA, M., ALLFREY, V., and MIRSKY, A.: The relationship between RNA synthesis and loop structure in lampbrush chromosomes. *Proc Nat Acad Sci USA* 49: 544-551, 1963.
- (25) MILLER, O. L.: Extrachromosomal nucleolar DNA in amphibian oocytes. *J Cell Biol* 23: 60A, 1964.
- (26) KEZER, J.: Unpublished observations quoted in Macgregor, H. C., The role of lampbrush chromosomes in the formation of nucleoli in amphibian oocytes *Quart J Micr Sci* 106: 215-228, 1965.

DISCUSSION

Brown: Can you correlate these results with the previous base composition studies that you made with Edström (Edström and Gall, *J Cell Biol* 19: 279-284, 1963) where you showed that the nuclear sap RNA has a nonribosomal base composition? Also, didn't the over-all labeling of chromosomal versus nonchromosomal RNA suggest that there were about equal numbers of counts in chromosomal RNA versus nucleolar and nuclear sap RNA during the lampbrush stage?

Gall: Let me answer the second question first. Autoradiographs of the chromosomes give a misleading picture of the relative amounts of radioactivity because we purposely exclude the nuclear sap; that is, we try not to precipitate nuclear sap onto the chromosomes when we make an autoradiograph. If one precipitates the nuclear sap in making a chromosome preparation, then one sees that the majority of the radioactivity is in the sap. In the base ratio analyses made by Edström, the nuclear sap had a composition which was variable from one preparation to the next, in contrast to the nucleoli and the chromosomes which gave consistent base ratio results. For some reason there seems to be more degradation or perhaps intrinsic variability of the sap RNA.

Pavan: When you showed the chromosomes with incorporated uridine, it appeared that there was a higher concentration of grains over the chromomeres than over the loops. How do you explain this?

Gall: I don't believe there is actual incorporation in the chromomeres of the chromosomes. You must remember that the chromosomes are dried down onto a slide, and the loops, which in the unfixed condition extend more or less in all directions from the main axis of the chromosome, collapse onto the main axis. So I think all of the incorporation is in the loops.

Vincent: Were your sucrose gradient curves done with added carrier or without?

Gall: Usually with carrier. It depended on the number of cells used. It wasn't necessary in all cases.

Vincent: Did you ever find any indication of a peak in what is normally considered to be the messenger region, that is between 8S and 16S RNA?

Gall: No, we never have found a peak in this region (*cf* text-figs. 1-3, this paper). The few times I have tried to extract RNA from the chromosomes only, I obtained a heterogeneous distribution throughout this range. But I'm not at all satisfied with these experiments.

Vincent: I ask these questions because we have found that messenger RNA will frequently aggregate with 18S RNA in velocity centrifugation at a relatively high concentration of applied RNA, particularly when the total sedimentation distance is minimal. Both of these conditions appear to be present in your experiments. In addition, the very high 28S to 18S RNA ratio suggests that breakdown of 28S RNA may have occurred.

Gall: This certainly could be true. However, I don't think there is any degradation of the radioactive 28S RNA because in long-term incubations the radioactivity profile is exactly coincident with the optical density.

Mandel: I do not think it can be concluded that 45S RNA contains only ribosomal RNA. We have evidence that the fraction sedimenting at 45S is heterogeneous and contains DNA-like RNA as well as ribosomal-like RNA. It is possible to distinguish between the two by treatment with actinomycin D. We have done our experiment on a plasmocytoma [*Bull Soc Chim Biol (Paris)*, in press]. We found 45S RNA with a high GC ratio in control animals. After injection of a small amount of actinomycin which inhibits the synthesis of ribosomal RNA, there is still a small peak of 45S RNA which has a DNA-like base ratio. Of course, the size of the messenger seems to be too large for any protein. However, in experiments concerning hemoglobin synthesis, it also appears that the messenger is much too big compared to the polypeptide chain which is produced. This might be explained by a polycistronic transcription followed by a degradation producing a

messenger specific for the polypeptide [Scherrer and Marguad, Bull Soc Chim Biol (Paris), in press].

Gall: I won't comment on that because I'm sure there will be discussion later on the nature of 45S RNA.

Penman: I hesitate to anticipate the data I will present later, but questions have come up. First of all, I'm very gratified to find the absence of 18S RNA from the nucleus of another cell type, using a very different method of preparation than we have used with the HeLa cell. Second, I want to comment about the nature of 45S RNA. If I do careful kinetics, and separate nucleoli, I find there is a 45S RNA in the nucleoli that is ribosomal precursor only. In the nucleoplasm there is RNA that runs from 10S to perhaps 80S, which is heterodisperse and rapidly labeled. There is as much of this RNA as ribosomal precursor RNA in the HeLa cell, though apparently not in the cell studied by Dr. Gall. When one looks at the 45S region from total nuclear RNA, there is both ribosomal precursor and heterodisperse, extranucleolar RNA. Regarding the question about what RNA is in the nucleoplasm, we have found both 35S and 45S RNA in the nucleolus, and a 28S ribosomal precursor RNA in the nucleoplasm. When one looks at total nuclear RNA, one sees the combination of the 35S and 28S which is not resolved on these gradients and which gives an apparent 30S sedimentation value. Depending on the cell, there will be both DNA-like, heterodisperse RNA and 28S ribosomal RNA precursor in the nucleoplasm, which probably gives rise to the differences in base composition ascribed to the nucleoplasm, depending on how much of this 28S is in transition from the nucleolus to the cytoplasm.

Pelling: Have you done density gradients of the RNA of whole cells? If so, is the label of the 28S and 18S RNA peaks in those gradients at any time proportional to the optical density? I ask this question because we have started density gradient studies on *Chironomus* salivary gland cells. In this material there is some indication that the relationship between the labeled 28S and 18S RNA peaks is not always constant. In pulse-labeling experiments, the relative height of the 18S RNA peak steadily increases with time and finally (24 hours after the uridine pulse) it is remarkably higher than the 28S RNA peak (unpublished data).

Gall: Actually we haven't done whole cells. We have used isolated nuclei and isolated cytoplasm.

RNA Metabolism in the HeLa Cell Nucleus and Nucleolus^{1, 2}

S. PENMAN,^{3, 4} I. SMITH, E. HOLTZMAN, and H. GREENBERG,^{4, 5} *Massachusetts Institute of Technology, Cambridge, Massachusetts, and Albert Einstein College of Medicine, New York, New York*

SUMMARY

A method of preparing a nuclear fraction from HeLa cells is described. The fraction does not contain an appreciable amount of the 16S RNA component of ribosomes and thus appears to be free from cytoplasmic contamination. All of the early precursors to cytoplasmic ribosomal RNA are contained in the nuclear fraction. Sedimentation analysis of the RNA extracted from the nuclear fraction shows a peak in optical density of 45S and a larger peak that sediments about 30S. The RNA in the 30S region is shown to be polydisperse and contains 35S and 28S RNA. Previous work has shown that the initial event in the formation of ribosomal RNA is the synthesis of 45S RNA. It is shown here that the next step is the cleavage of 45S RNA to form 16S RNA which emerges immediately into the cytoplasm and 35S RNA which remains in the nucleus. The 35S

RNA undergoes a transition to 28S and then enters the cytoplasm. The cleavage of 45S to 35S and 16S RNA proceeds in the presence of actinomycin but the subsequent behavior of the 35S RNA is abnormal. Experiments in which radioactive label is chased show that there is no high molecular weight RNA permanently associated with the nucleus. A method of digesting the nuclear fraction using high ionic strength buffer and deoxyribonuclease is described. A particulate fraction is obtained which morphologically resembles nucleoli and which has the 45S and 35S ribosomal RNA precursors. A preliminary experiment on the methylation of RNA indicates that the 45S ribosomal precursor is the only high molecular weight RNA which is methylated in a short pulse.—*Nat Cancer Inst Monogr* 23: 489–512, 1966.

THIS REPORT describes a method of preparation which makes possible a virtually complete separation of the nucleus and cytoplasm of HeLa cells. The nuclear fraction can be further divided into nucleolar and

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² This work was supported by grant GB2679 from the National Science Foundation, grants E-380 and PF-297 from the American Cancer Society, Inc., grant DRG-872XT from the Damon Runyon Memorial Fund, and Public Health Service grants CA-08416-01 and CA-06756 from the National Cancer Institute.

³ During the major part of this work, S. Penman was a recipient of a Career Development Award of the Health Research Council of the City of New York.

⁴ Massachusetts Institute of Technology.

⁵ We would like to thank Maria Penman for her technical assistance in many laborious tasks. We would also like to express our appreciation to Dr. A. B. Novikoff for his advice and for the use of his electron microscope and other facilities and to Mr. J. Godrich for preparation of the photographs.

nucleoplasm fractions. The results of a detailed study of nuclear and nucleolar RNA metabolism are also described. Analysis of the RNA content of the nucleus shows a complete absence of the 16S RNA component of mature ribosomes. This finding is then used to establish the absence of contaminating cytoplasm from the nuclear preparation.

The nuclear preparation contains all of the early precursors to cytoplasmic ribosomal RNA. The absence of such precursors from the cytoplasmic fraction is used to establish the freedom from contamination of the cytoplasm by the nuclei. The nucleolar preparation contains all the 45S and 35S RNA that is precursor to cytoplasmic ribosomal RNA. The complete absence of these species of RNA from the nucleoplasm serves to establish its freedom from contamination by what is operationally defined as the nucleolar preparation. Conversely, the nucleoplasm contains a rapidly labeled, highly disperse RNA fraction that is largely absent from the nucleolar fraction. The preparation thus yields a separation between cell nucleolus, nucleus, and cytoplasm which is meaningful in terms of the partition of the cellular RNA.

The preparation is quite simple to obtain and completely reproducible with respect to the RNA obtained in each fraction. It can thus serve as the basis of an operational definition of nucleolus, nucleus, and cytoplasm, *i.e.*, fractions prepared according to the described method.

In addition, studies made with the electron microscope show that the nuclear preparation contains entities that resemble the intact cell nucleus and which appear free from any visible cytoplasmic contamination. The nucleolar preparation is composed largely of structures resembling, at least grossly, the nucleoli of the intact cell.

To obtain precise measurements of the amount of RNA in the nuclear fraction, a method permitting complete recovery of the nuclear RNA is necessary. Disruption of the nuclear structure results in formation of a nucleohistone gel. During extraction with phenol the gel forms an aggregate which traps some of the nuclear RNA. A method of digesting the nucleohistone gel while preserving the nuclear RNA was developed. The digestion procedure apparently leaves the nucleoli intact, and the nucleolar fraction is obtained by centrifugation of the nuclear digest before phenol extraction. In addition, a modification of the phenol extraction which facilitates recovery of 85 to 90% of the nuclear RNA is described.

The methods of fractionation described here are used to determine the site of methylation of the high molecular cellular RNA.

MATERIALS AND METHODS

Preparation of the nuclear fraction.—The cells used in these experiments were type 3 HeLa growing in suspension cultures. The methods of culture have been described previously (1). The nominal concentration of cells when growing is 4×10^5 /ml. The usual sample consisted of 4×10^7

cells. After labeling with a suitable radioactive precursor the cells were harvested by centrifugation in the International PR-2 for $1600 \times g/\text{minute}$. The cells were then washed once with cold Earle's saline (2) and deposited by centrifugation.

The preparation was made at 0–4 C. The cell pellet obtained after washing was resuspended in 2 ml of the hypotonic buffer RSB (0.01 M NaCl, 0.01 M Tris, 0.0015 M $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, pH 7.4). The cells were allowed to swell for 10 minutes and then were broken with a precision bore, stainless-steel ball homogenizer. The clearance between the ball and the bore of the homogenizer was quite critical. A clearance of 0.002 inches was found to give a high efficiency of opening cells with a minimum of nuclear rupture.

The nuclei were removed by centrifugation for $1600 \times g/\text{minute}$ and the supernatant was saved. The nuclei were then resuspended in 2 ml of RSB by vigorous pipetting and deposited by centrifugation. The supernatant was combined with the previous one. The nuclei were again resuspended in 2 ml of RSB.

At this point, examination of the nuclear preparation by phase microscopy showed the nuclei to be relatively free of observable cytoplasmic tabs. There was a contamination of unbroken cells which varied from 1 to 5%. The following procedure removed the remaining cytoplasm and whole cells.

A mixture was made of one part of a 10% w/w solution of the ionic detergent, sodium deoxycholate and two parts of a 10% w/w solution of the nonionic detergent Tween-40 (polyoxyethylene sorbitol monopalmitate). Three tenths of a milliliter of this solution was added to the nuclear suspension and the suspension shaken with a vortex mixer for 3 seconds. The nuclei were then deposited by centrifugation and the supernatant was added to the previous ones. The combined supernatants were designated the cytoplasmic fraction. The mixture of detergent solutions must be stored frozen since nonionic detergents will support the growth of mold.

Digestion of the nuclei.—The pellet obtained after centrifugation of the detergent-treated nuclei cannot be readily dispersed since the nuclei show a strong tendency to aggregate after the detergent treatment. This pellet cannot be directly extracted with phenol since an unmanageable mass of DNA which traps much of the nuclear RNA is obtained. The pellet was disrupted by digestion with deoxyribonuclease in high ionic strength high salt buffer (0.5 M NaCl, 0.05 M MgCl_2 , 0.01 M Tris-HCl, pH 7.4).

Two ml of high salt buffer was added to the nuclear pellet and the preparation was warmed to room temperature. Approximately 100 μg of electrophoretically purified deoxyribonuclease was added. The mixture was stirred vigorously. The nuclear pellet gradually dissolved to yield a viscous solution. The preparation was allowed to remain at room temperature until it was no longer viscous and there were no visible clumps. Two minutes of treatment with deoxyribonuclease was usually sufficient. If total nuclear RNA was to be extracted, sodium dodecyl sulfate (SDS) was then added to a concentration of 0.5%, and a solution of sodium salt

of EDTA at pH 7.4 was added to a concentration of 0.1 M. The mixture was somewhat turbid because SDS was not completely soluble at these salt concentrations and pH.

Preparation of the nucleolar fraction.—The digestion of the nuclear fraction with deoxyribonuclease was carried out as described above. Before the addition of SDS and EDTA, however, the digest was chilled and then centrifuged for $50,000 \times g$ /minute in a 15 ml tube in the Servall refrigerated centrifuge. The supernatant was decanted and termed nucleoplasm. The nucleoplasm was then treated with SDS and EDTA as described for whole nuclei. The pellet, which is termed the nucleolar fraction, was resuspended by stirring at 37 C in SDS buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCl, pH 7.4, 0.5% SDS). The fraction was then phenol-extracted as described below.

Extraction of the nuclear and nucleolar RNA.—The procedure described here was a modification of the hot phenol-SDS method described by Scherrer and Darnell (3). It permits complete recovery of the nuclear RNA by preventing loss of RNA complexed with denatured protein and SDS into the phenol.

Two ml of phenol was added to the digested nuclei and the mixture shaken on a vortex mixer. The mixture was then heated to 60 C and shaken again.

Two ml of chloroform containing 1% isoamyl alcohol was then added, and the mixture was reheated to 60 C and shaken on a vortex mixer. The mixture was then centrifuged at room temperature in an International PR-2 centrifuge at 2800 rpm for 2 minutes. The preparation then contained a phenol-chloroform phase below, an aqueous phase above, and a large flocculent precipitate at the bottom of the aqueous phase. This precipitate contained much of the nuclear RNA and would have sunk into the phenol phase if the density of the lower phase had not been increased by addition of chloroform. The lower phase was turbid from undissolved SDS. Reheating the preparation to 60 C removed this turbidity.

The phenol-chloroform phase was removed with a Pasteur pipette. The precipitate was left in the aqueous phase. The procedure was then repeated 3 times using the chloroform-isoamyl alcohol mixture alone. At this point no flocculent precipitate remained. There was a thin interfacial layer which was presumably composed of the denatured proteins of the nucleus and was free of complexed SDS.

The aqueous phase from the last extraction was added to 2 volumes of 95% ethanol and the mixture allowed to stand for 1 hour at -20 C. The resulting precipitate was centrifuged at 0 C in a Servall model SS-1 for 15 minutes at 15,000 rpm. The precipitate was then resuspended in 1 ml of SDS buffer. Some preparations were resuspended in 10X RSB buffer and treated with 50 μ g of electrophoretically purified deoxyribonuclease to reduce the size of the high molecular weight DNA that remained. SDS was then added to 0.5% before centrifugation.

Sucrose density gradient analysis was performed with sucrose-SDS as previously described (4).

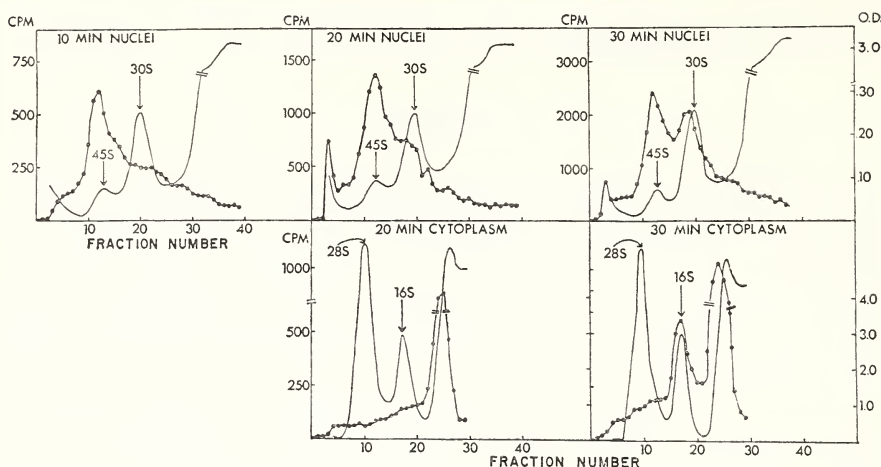
RESULTS

The electron microscope pictures in figures 1 through 9 show the appearance of the nucleus of the HeLa cell at the various steps in the preparation of the nuclear fraction. The most significant morphological change was perhaps the dispersion of the chromatin material when the cells were swollen in hypotonic buffer. The removal of the outer nuclear membrane with the attached perinuclear ribosomes by the mixed detergents is also clearly shown.

Results of sucrose density gradient analysis of the RNA from the nuclear fraction are shown in the upper section of text-figure 1. The optical density profile of the nuclear RNA shows two peaks. The more rapidly sedimenting RNA has a sedimentation constant of approximately 45S. The less rapidly sedimenting species of RNA has a sedimentation constant close to that of 28S ribosomal RNA. Further analysis showed that this material was not a single species and that its nominal sedimentation was slightly greater than 28S. For purposes of identification it will be referred to as 30S RNA. It should be noted that no RNA species corresponding to 16S ribosomal RNA was apparent in the optical density profile. This is even more clearly demonstrated in the nuclear RNA fractions shown in text-figure 2. The centrifugation was more extensive in this experiment and the 16S region was moved beyond the region of degraded DNA at the top of the gradient. It is estimated that less than 0.5% of the amount of 16S RNA present in the cytoplasmic fraction was found in the nuclear preparation. The absence of a significant amount of 16S RNA is the basis for claiming an absence of structures corresponding to cytoplasmic ribosomes in the nuclear fraction. Generally the amount of observable 16S material serves as a convenient method for quantifying the degree of purity of the nuclear fraction, *i.e.*, the freedom from cytoplasmic contamination.

The sedimentation profile of the RNA of the cytoplasmic fraction is also shown in the lower section of text-figures 1 and 2. The cytoplasmic RNA was deproteinated simply by adding SDS, and the expected 16S and 28S species of RNA were obtained. The optical density at the top of the gradients is due to sRNA and protein.

The results of labeling cells for short periods with radioactive uridine are also shown in text-figures 2 and 3. A large fraction of the nuclear RNA labeled during a 10-minute exposure to radioactive uridine is seen to coincide in sedimentation with the 45S peak observed in the optical density profile. This is the ribosomal RNA precursor first described by Scherrer and Darnell (3, 5). A large amount of polydisperse RNA is also labeled. This polydisperse RNA may be in part composed of partially synthesized 45S molecules, but there is clearly another species of RNA



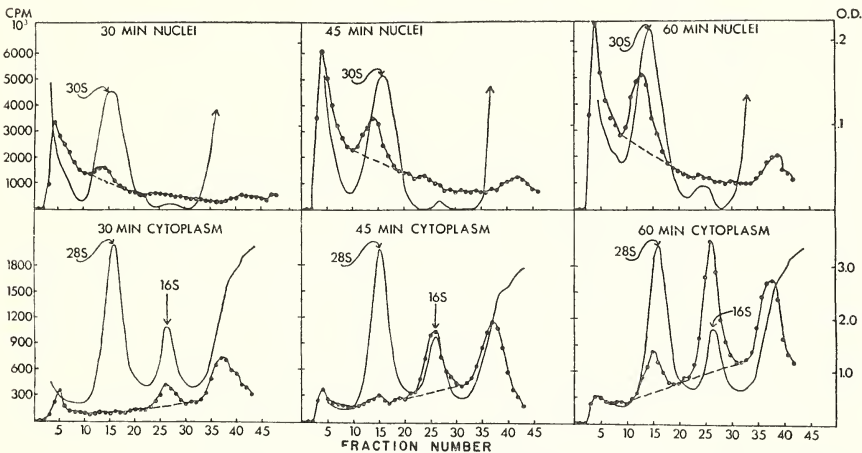
TEXT-FIGURE 1.—Pulse-labeled RNA in HeLa cells. Cells growing at a concentration of 4×10^5 /ml were concentrated to 2×10^6 /ml. C^{14} -uridine of a specific activity of $27 \mu\text{c}/\mu\text{mole}$ was added at a concentration of $0.05 \mu\text{c}/\text{ml}$. Samples consisting of 4×10^7 cells were removed at the indicated times and the incorporation was stopped by pouring over crushed, frozen Earle's saline. Nuclei and cytoplasm were separated as described in Materials and Methods. The nuclei were digested with electrophoretically purified deoxyribonuclease in high salt buffer, and the RNA was extracted with phenol as described in Materials and Methods. The cytoplasm was made 0.5% in sodium dodecyl sulfate. The RNAs from both fractions were layered on 15 to 30% sucrose gradients and centrifuged in the SW25 rotor for 16 hours at 17,000 rpm. The gradients were eluted through a continuously recording spectrophotometer, set to measure the optical density at $260 \text{ m}\mu$, and collected in fractions. The fractions were made 10% in trichloroacetic acid and the precipitate was collected on membrane filters. The filters were dried and counted in a thin window gas flow counter. The data obtained for the cytoplasmic fraction have been corrected since only one third of the fraction was layered on the sucrose gradient. *Solid line represents optical density; dotted line, radioactivity.*

present which is as yet uncharacterized. Its sedimentation characteristics make it unlikely that it is identical to the messenger RNA obtained in the cytoplasm. It is shown later that the polydisperse nuclear RNA is found largely in the nucleoplasm, external to the nucleoli.

After 30 minutes of incorporation, a peak was observable in the region corresponding to 35S RNA. This was the 35S ribosomal precursor observed by Scherrer and Darnell (3, 5) and Rake and Graham (6). The 35S peak in radioactivity was not coincident with the optical density profile of the 30S peak.

The 35S peak in radioactivity appears in the nucleus at the same time the 16S RNA initially appears in the cytoplasm. This suggests that transformation of 45S RNA to 35S RNA in the nucleus is accompanied by the formation of 16S RNA. The 16S RNA apparently emerges immediately into the cytoplasm.

The results of uridine incorporation in the cell for 30, 45, and 60 minutes are shown in text-figure 2. The RNA in these gradients had been sedimented further than in the gradients shown in text-figure 1 in order to display in greater detail the RNA in the 30S to 35S region. Again the distinct peak of radioactivity in 35S region sediments ahead of the peak in optical density. The amount of radioactivity in the 35S region increases with time. At 60 minutes after the addition of uridine, the first radioactivity in the 28S region is seen in the cytoplasm.

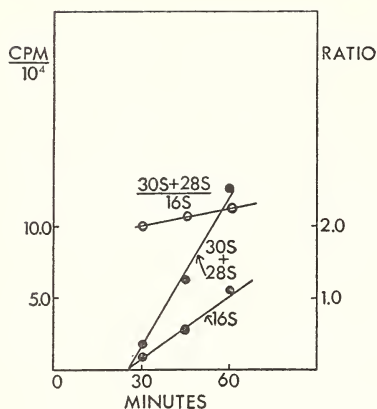


TEXT-FIGURE 2.—Pulse-labeled RNA in HeLa cells. The experimental details are the same as those described in text-figure 1, except for the times indicated in the diagrams and the centrifugation schedule which was for 16 hours at 22,000 rpm. Solid line represents optical density; dotted line, radioactivity.

The 35S RNA in the nucleus appears to serve as a precursor to 28S RNA in the cytoplasm. A minimum of 30 minutes was required for 35S RNA to pass through the nuclear stage and emerge into the cytoplasm. During this time, its sedimentation decreased to 28S.

An estimate was made of the total radioactivity in the peak associated with the 35S nuclear RNA and in 16S cytoplasmic RNA at the times shown in text-figure 2. The results are shown in text-figure 3. The concurrent appearance of radioactively labeled 16S RNA in the cytoplasm and 35S RNA in the nucleus noted in text-figure 2 is clearly demonstrated here since the graphs depicting the amount of radioactivity in each species both extrapolate back to a common origin at 25 minutes.

The ratio of the total radioactivity found in 35S RNA to that in 16S RNA in text-figure 2 is also plotted. At 60 minutes some radioactivity appeared in 28S cytoplasmic RNA. Since it appeared that 35S RNA and 28S RNA were related species of RNA, the radioactivity in 28S RNA was added to that in 35S RNA. It can be seen that the ratio of total radioactivity of 35S to 16S RNA is approximately constant during the time of



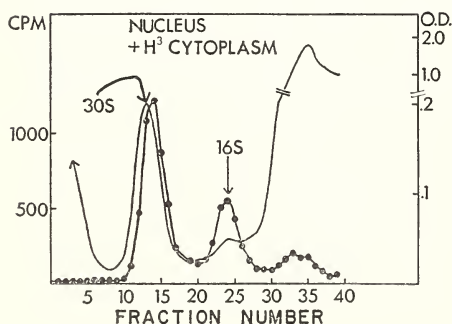
TEXT-FIGURE 3.—Appearance of 16S and 35S RNA. Estimates were made from the data in text-figure 2 of the amount of radioactivity in the 16S peak in the cytoplasm and the 30S region in the nucleus. The small amount of radioactive 28S RNA in the cytoplasm was added to that in the 35S RNA. Also the ratio between the two classes of RNA is shown.

measurement. The ratio is also very close to 2. When the radioactivity in 28S and 16S RNA from mature ribosomes labeled with uridine is measured, the ratio is also found to be close to 2.

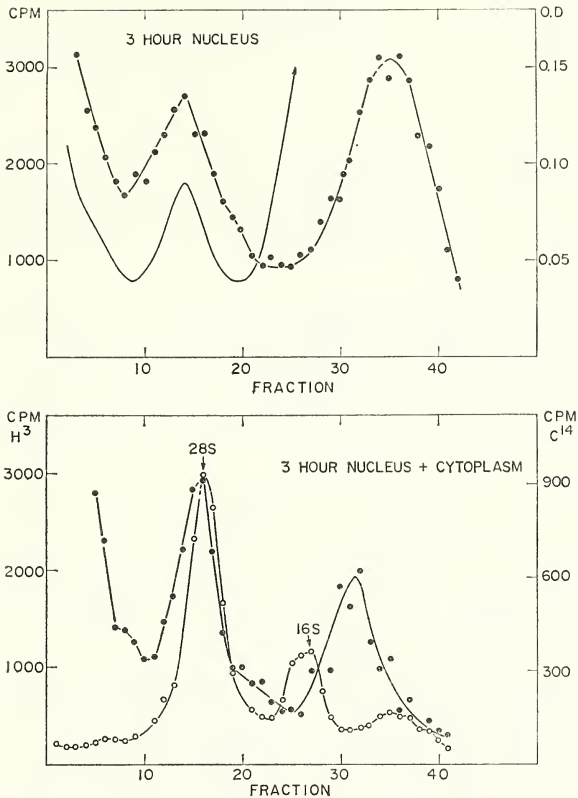
It has been shown that the labeled RNA, which first appears in the nucleus in the 35S region, sediments ahead of the peak visible by optical density in a sucrose gradient of nuclear RNA. The following experiments show that the peak in optical density, which we have termed 30S RNA, sediments faster than the 28S RNA from cytoplasmic ribosomes.

A cytoplasmic extract was prepared from cells that had been labeled for 24 hours with tritiated uridine. A small amount of this cytoplasm was added to a nuclear preparation from unlabeled cells. The mixture was extracted as described and the RNA subjected to sucrose density gradient analysis. The amount of cytoplasm was insufficient to contribute significant optical density. Thus the optical density profile of the sucrose gradient was due to nuclear RNA, whereas the radioactivity was derived solely from cytoplasmic ribosomes.

The results are shown in text-figure 4. The optical density peak of the nuclear RNA sediments a small but significant amount ahead of the peak in radioactivity corresponding to 28S cytoplasmic RNA. It may be noted that the cytoplasmic 16S RNA has been recovered, which shows that the



TEXT-FIGURE 4.—Sedimentation characteristics of nuclear and cytoplasmic RNA. Cytoplasm was prepared from cells highly labeled with H^3 -uridine; 0.25% of the cytoplasm from 4×10^7 cells was added to a nuclear preparation from a similar number of cells. The mixture was digested with deoxyribonuclease in high salt buffer and then phenol-extracted. Sucrose gradient analysis was as described in text-figures 2 and 3. Solid line represents optical density; dotted line, radioactivity.



TEXT-FIGURE 5.—Sedimentation analysis of the 30S nuclear RNA. *Upper*: optical density and radioactivity profile obtained with density gradient sedimentation analysis of the nuclear RNA obtained from 4×10^7 cells. The cells were labeled for 3 hours with C^{14} -uridine at a concentration of $3 \mu\text{g/ml}$ and a specific activity of $5 \mu\text{c}/\mu\text{mole}$. Centrifugation was for 16 hours at 22,000 rpm in the SW25.1 rotor. *Lower*: radioactivity profile obtained from a mixture of C^{14} -labeled nuclear RNA obtained as above and cytoplasmic RNA obtained from cells labeled for 24 hours with H^3 -uridine. For this experiment, the cytoplasmic RNA was extracted with phenol and chloroform as described in Materials and Methods. *Solid line* represents optical density; *dotted line*, radioactivity.

absence of 16S RNA from the nuclear preparations is not due to a gross artifact.

Since the difference in sedimentation between the nuclear 30S and the cytoplasmic 28S is small, their relative sedimentation constants were measured in a slightly different way. Text-figure 5 shows the sedimentation of nuclear RNA from cells labeled with uridine- C^{14} for 3 hours. The

optical density profile and the radioactivity are seen to coincide. This is expected since the optical density profile of RNA represents the steady state distribution as should the radioactivity after a sufficiently long period of labeling.

Also shown in text-figure 5 is the result of a double labeling experiment where nuclear RNA of cells labeled for 3 hours with C^{14} -uridine is mixed with cytoplasmic RNA of cells labeled with tritiated uridine for 24 hours. The noncoincidence of the nuclear 30S and the cytoplasmic 28S may also be seen here.

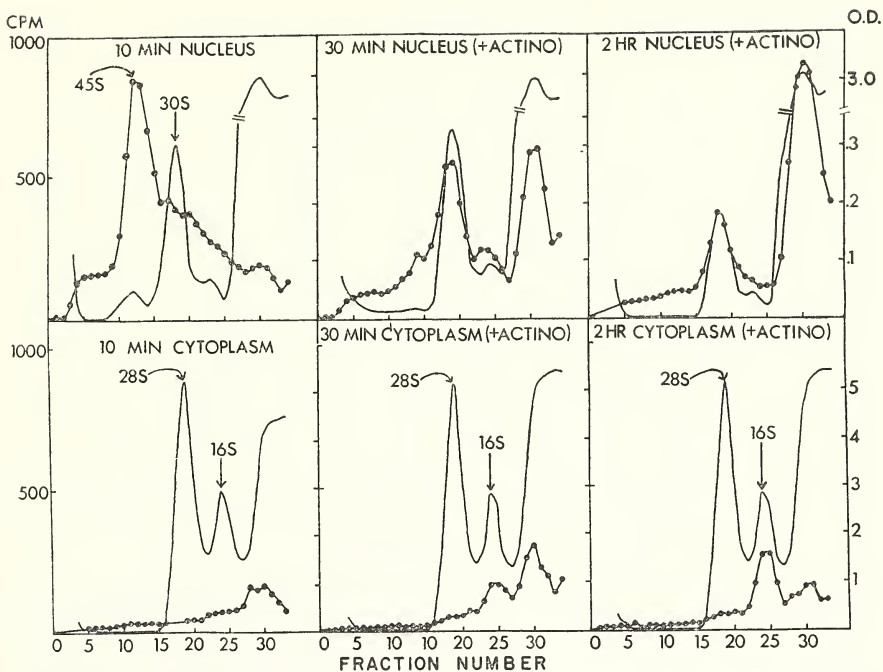
The effect of actinomycin on pulse-labeled RNA was investigated. It has been shown that, in the presence of actinomycin, the 45S RNA labeled in a short pulse undergoes a transition to material with a smaller sedimentation coefficient (3). It has also been shown that actinomycin interfered with the transfer of ribosomal RNA from the nucleus to the cytoplasm (7).

The results of the experiments using actinomycin are shown in text-figure 6. Cells were labeled with radioactive uridine. The pattern of radioactivity obtained after 10 minutes was essentially the same as that shown in text-figure 2. At 10 minutes, actinomycin was added to a concentration of 5 μ g/ml. This concentration was sufficient to completely inhibit all RNA synthesis within 5 minutes.

RNA was extracted from cells at 30 minutes and 2 hours after the addition of label and analyzed on sucrose gradients. At 30 minutes, the 45S RNA, as measured by optical density and radioactivity, had almost disappeared from the nucleus. At 2 hours the radioactivity and optical density in the 30S to 35S region decreased significantly. There was, however, no appearance of 28S RNA in the cytoplasm.

Short periods of incorporation of radioactive RNA precursor do not show whether there is stable high molecular weight RNA permanently associated with the nuclear fraction. An experiment was designed to measure the amount of stable RNA in the nucleus. Cells were labeled with uridine for 24 hours. The label was then chased for 72 hours with an excess of cold uridine to avoid continuing incorporation of radioactivity from the nucleotide pools of the cells. Thymidine and deoxycytidine were present in the medium to minimize the incorporation of radioactive label into DNA. The cells grew normally during the experiment.

Despite the addition of nonradioactive deoxypyrimidines to the medium, there is always considerable incorporation of radioactive label into DNA during long label periods. To distinguish between radioactive label contained in RNA and that contained in incompletely digested DNA, the sucrose gradients obtained in this experiment were collected in duplicate fractions. One set of fractions was precipitated immediately and the other subjected to mild alkaline hydrolysis before precipitation with trichloroacetic acid. Since the radioactivity incorporated into DNA is resistant to the conditions of hydrolysis used, the RNA content of the

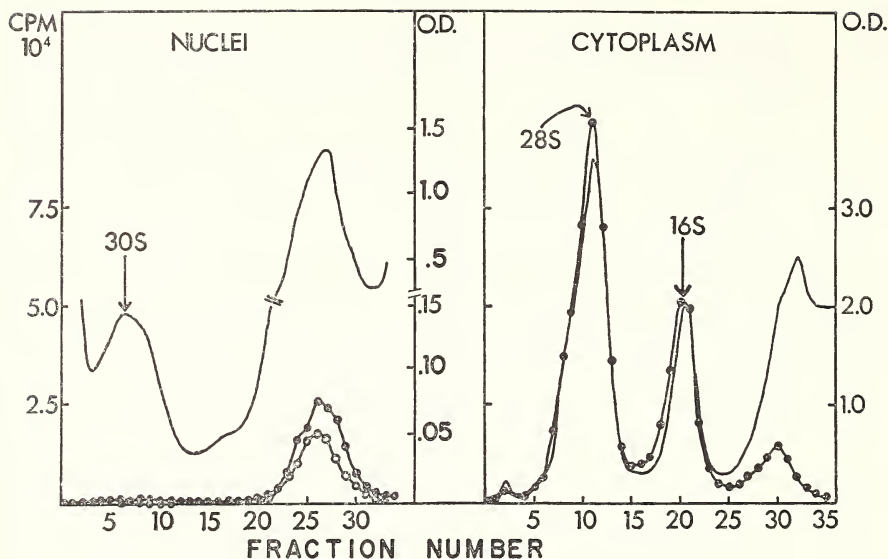


TEXT-FIGURE 6.—Pulse-labeled RNA in actinomycin-treated cells. Cells were labeled as described in text-figure 2 except that actinomycin was added after 10 minutes of incorporation. RNA was prepared as previously described except that centrifugation was carried out with the 6 place SW25.3 rotor in the Spinco model L-2. Centrifugation was at 19,000 rpm for 16 hours. *Solid line* represents optical density; *dotted line*, radioactivity.

gradients is obtained by taking the difference between the two sets of fractions. Appreciable incorporation into DNA does not occur for short labeling periods because uridine is not directly incorporated into DNA but must first be converted to a deoxypyrimidine.

The results of the long label and chase are shown in text-figure 7. It can be seen that the nuclear fraction contains no appreciable amount of high molecular weight RNA as compared to the cytoplasmic fraction. The 45S material is sedimented to the bottom of the centrifuge tube to display the 16S region in greatest possible detail. A similar experiment in which the 45S material was retained in the gradient also showed complete absence of stable RNA in the nuclear fraction.

The next experiments to be described show that the nuclear preparation can be further subdivided into nucleolar and nucleoplasm fractions. The high ionic strength buffer used to digest the nuclei had been previously found to leave the structure of mature ribosomes intact (Penman, unpublished observation). In fact, the buffer had been originally developed to permit the observation of polyribosomes in the presence of considerable nuclear material. After the nuclear fraction was digested, a particulate

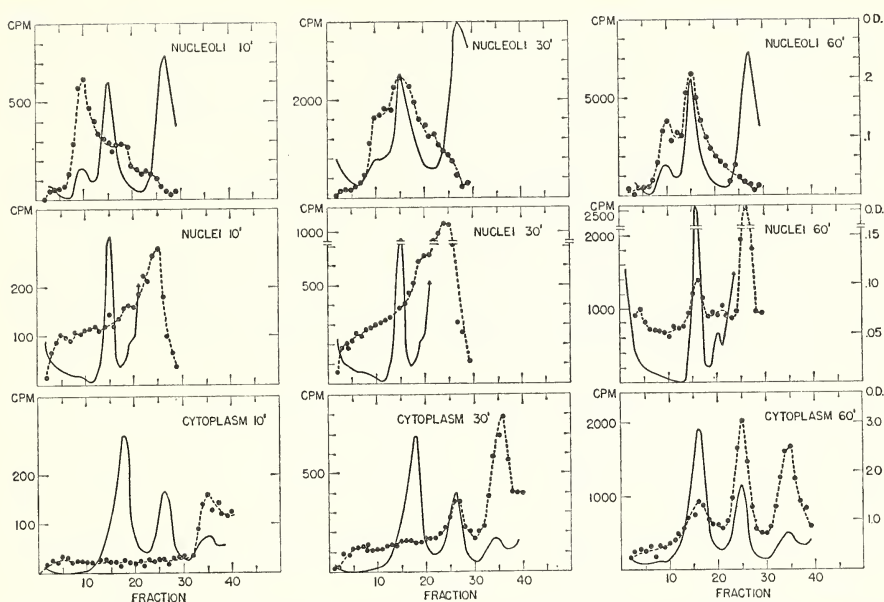


TEXT-FIGURE 7.—Analysis of stable RNA associated with the HeLa cell nucleus. Cells, 2×10^7 , were labeled with approximately $2 \mu\text{c}$ of C^{14} -uridine for 24 hours. Unlabeled thymidine and deoxycytidine were added to a concentration of 10^{-6} M . At the end of the labeling period, the cells were diluted to maintain a constant concentration for growth, and the medium was made 10^{-5} M in unlabeled uridine, cytidine, thymidine, and deoxycytidine. The cells were grown for 3 days, during which time the rate of growth was normal. RNA was prepared from nucleus and cytoplasm as previously described in text-figure 2 and a sucrose gradient analysis was performed. Centrifugation was in the SW25.1 rotor for 16 hours at 22,000 rpm. The fractions collected from the nuclear RNA gradient were in duplicate. One set of fractions was assayed for acid-precipitable radioactivity immediately; the other set was made 0.3 N in NaOH and incubated at 35°C overnight before being assayed for acid-precipitable radioactivity. Total radioactivity (—•—•—); alkali stable radioactivity (—o—o—).

component was observed both by light scattering and in the phase microscope. It seemed possible that the organized structure involved in ribosome production, the nucleolus, had survived the digestion in high salt buffer and could be easily sedimented.

Figures 8 and 9 show the composition of the nucleolar fraction as seen by the electron microscope. The low-power view shows that the principle constituents are structures morphologically resembling the nucleoli of intact cells. It is apparent that there is some contamination from what appears to be clumped chromatin material. The high-power view suggests that much of the structure of the intact cell's nucleolus has been preserved.

The RNA obtained from the nucleoli, nucleoplasm, and cytoplasm of cells pulse labeled with a radioactive uridine is shown in text-figure 8. It can be seen from both optical density and incorporated radioactivity that the peak corresponding to 45S ribosomal precursor is contained entirely



TEXT-FIGURE 8.—HeLa cells were labeled with $0.1 \mu\text{C}/\text{ml}$ of C^{14} -uridine for the indicated times. Nucleolar, nuclear, and cytoplasmic fractions were prepared as described in Materials and Methods. The preparations were phenol-extracted as previously described. Sucrose gradient analysis was performed with an SW25.3 rotor in the Spinco model L-2 ultracentrifuge. The nucleolar and nuclear gradients were centrifuged for 21,000 rpm for 16 hours at 26 C. The cytoplasm gradients were centrifuged at 24,000 rpm for 16 hours.

in the nucleolar fraction. It is the principle species of RNA labeled in 10 minutes in the nucleolus. There is a small shoulder to the more slowly sedimenting side of the 45S peak. Recent experiments suggest strongly that this is another species of rapidly labeled RNA whose function has not yet been determined.

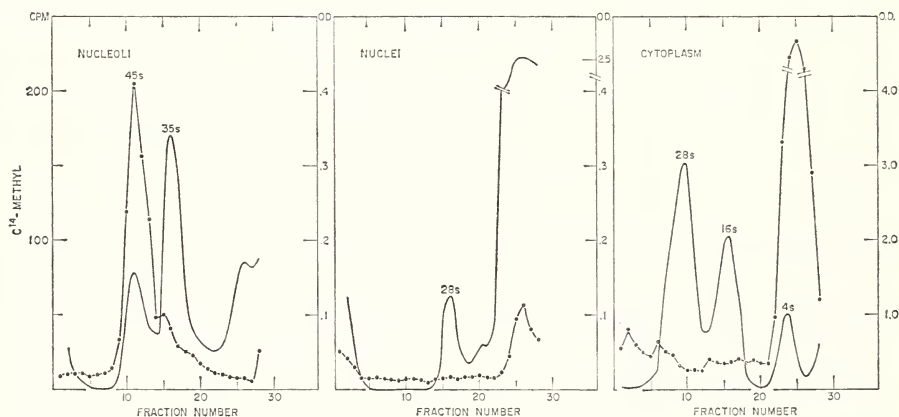
The fraction-labeled nucleus, which consists of the extranucleolar RNA and which has been referred to as the nucleoplasm, is seen to contain most of the polydisperse, rapidly labeled RNA. This species of RNA can be seen underlying the 45S peak in the gradient's total nuclear RNA shown in text-figure 1. There is also a small peak visible in optical density in the region of 28S. Other experiments have shown that, in fact, the sedimentation coefficient of the RNA in this peak is identical with that of the 28S RNA from mature ribosomes. The 28S RNA in the nucleoplasm does not arise from contamination by cytoplasmic ribosomes since there is no accompanying 16S ribosomal RNA and the kinetics of labeling show this RNA to be intermediary between nucleolar 35S and cytoplasmic 28S RNA.

After 30 minutes of incorporation, two peaks of radioactivity can be seen in the nucleolar fraction, the 45S and what we have termed for identification "the 35S." It may be noted that the noncoincidence between the ear-

liest radioactivity in the 35S region and the peak in optical density, seen when total nuclear RNA is examined, is no longer apparent when RNA is extracted from the nucleolar fraction alone. The 28S RNA in the nucleoplasm still contains no radioactivity other than that of the polydisperse RNA that happens to coincide in sedimentation.

After 60 minutes of labeling, the 28S RNA in the nucleoplasm is seen to contain radioactivity. The 28S RNA in the cytoplasm is also labeled by this time but at a much lower specific activity.

The separation of cellular RNA described in this report suggests another type of experiment designed to determine what type of RNA is methylated and where the site of methylation is to be found.



TEXT-FIGURE 9.—HeLa cells were concentrated to 4×10^6 cells per ml in methionine-free medium. C^{14} -methyl methionine ($10 \mu\text{c}/\mu\text{mole}$) was added at a concentration of $0.5 \mu\text{c}$ per ml. Incorporation was for 10 minutes, and then the cells were chilled with crushed, frozen Earle's saline. Nucleolar, nuclear, and cytoplasmic fractions were prepared as described in Materials and Methods. In this experiment, all fractions including the cytoplasm were extracted twice with phenol so as to completely remove methionine present in protein. Sucrose gradient analysis was as described.

Cells were labeled for 10 minutes with C^{14} -methyl methionine. The RNA was extracted with phenol from the nucleolar, nuclear, and cytoplasmic fractions. The results of density gradient sedimentation analysis are shown in text-figure 9. The radioactivity in the slowly sedimenting regions of the gradients was presumably due to methylated sRNA, DNA, and sRNA charged with methionine. Of the rapidly sedimenting species of RNA, only the 45S peak in the nucleolus contains significant radioactivity.

DISCUSSION

Recent research has established the following facts about the RNA metabolism of HeLa cells. When the sedimentation pattern of total cell

RNA which has been labeled for a short time is examined, most of the incorporated radioactivity is found in the 45S component which has a base composition resembling that of ribosomal RNA (3,5,6). If the labeling period is extended, the radioactivity is found to be distributed in 16S, 28S, and 35S RNA. After very long periods of incorporation, the radioactivity is found principally in 16S and 28S RNA. These results show that the initial event in the synthesis of ribosomal RNA is the formation of a large molecule (45S) which is eventually processed to form the 16S and 28S components of ribosomes.

It is relatively straightforward to prepare a cytoplasmic fraction from HeLa cells free from nuclear contamination. With this preparation it is possible to follow the emergence of RNA from the nucleus shortly after the beginning of incorporation of radioactive precursor to RNA (8). In the cytoplasm, the messenger RNA associates with pre-existing ribosomes forming polyribosomes. About 30 minutes later, the 16S ribosomal component begins to appear and 30 minutes after that the 28S component can be observed (9). These observations have been interpreted to mean that, unlike messenger RNA, ribosomal RNA is delayed in the nucleus after synthesis, presumably because of the time required for its eventual incorporation into a functional ribosomal subunit. Part of the delay can be accounted for by the time required for the original 45S RNA to complete its transition to 16S and 28S components.

The nuclear preparation described in this work permits the examination of the nuclear events of RNA metabolism without cytoplasmic contamination, thus extending previous results and allowing further investigation of the early events in ribosomal RNA synthesis.

The method for preparing a nuclear fraction described here consists of two principal steps: mechanical removal of most of the cytoplasm by shearing the cells in hypotonic buffer, and treatment with detergent to remove the remaining cytoplasm. The preparation is easy to obtain, reproducible with respect to the RNA it contains, and is composed of entities grossly resembling the nuclei of intact cells.

An important characteristic of this preparation is the absence of 16S RNA, indicating the absence of any structures corresponding to mature cytoplasmic ribosomes. The amount of 16S RNA in a nuclear preparation can thus be used as a measure of cytoplasmic contamination, just as the amount of early labeled ribosomal precursors in cytoplasmic preparation can be used as a measure of nuclear contamination.

Previous reports have shown that the initial event in the formation of ribosomal RNA is the synthesis of a species of RNA with a sedimentation coefficient of 45S. Our experiments strongly suggest that the next event is the separation of the 45S RNA into 16S RNA, which appears in the cytoplasm immediately, and a molecule with a sedimentation coefficient of approximately 35S, which remains in the nucleus. There are two independent observations supporting this interpretation of the data: (a) The 16S and 35S species of RNA first appear at the same time (about 25 min-

utes after the beginning of synthesis of radioactive RNA). (b) When actinomycin is added to cells 10 minutes after the beginning of the incorporation of radioactive label, RNA synthesis stops, and the 45S RNA present disappears with the accompanying formation of cytoplasmic 16S and nuclear 35S RNA.

The transition of 35S RNA to 28S RNA appears to take place without an extensive loss of material from the molecule. Evidence to support this conclusion is drawn from the fact that after short periods of labeling the ratio of radioactivity, which approximates the ratio of radioactivity of 16S RNA to 28S RNA, that can be determined would not permit the detection of a small change in the amount of radioactivity during the transition from 35S to 28S.

The radioactivity initially found in the 30S to 35S region of gradients of nuclear RNA sediments faster than the peak in optical density at 30S. After longer periods of incorporation, the radioactivity becomes coincident with the optical density. Since the 35S RNA is eventually transformed to 28S RNA, it appears very likely that both species are present in the nucleus and their overlapping sedimentation profiles give rise to a composite peak with a nominal sedimentation coefficient of 30S.

Our data on the transfer of ribosomal RNA from the nucleus to the cytoplasm agree with previous results obtained by the study of cytoplasm alone. Thus, it was previously shown that 16S RNA first appears in the cytoplasm in the form of a 45S ribosomal subunit about 25 minutes after the beginning of the incorporation of a radioactive precursor to RNA. 28S RNA first appears in the cytoplasm in a 60S ribosomal subunit about 60 minutes after the beginning of labeling (9). The agreement between earlier studies and the results shown here indicates the detergent treatment of the nuclei does not cause a gross loss of RNA from nucleus to cytoplasm. In particular, the observed immediate transfer of 16S RNA from the nucleus to the cytoplasm probably takes place in the live cell and is not an artifact of the method of preparation.

It is apparent from the sucrose gradients of nuclear RNA that there are rapidly labeled molecules of RNA which differ from ribosomal precursors. This RNA is polydisperse in sedimentation and can be seen underlying the peaks corresponding to the ribosomal precursor RNA in text-figures 2 and 3. The sedimentation characteristics of this RNA are quite different from the cytoplasmic messenger RNA which can be seen in the cytoplasmic fractions shown in text-figure 2. It is also present in an amount far greater than would be expected for RNA that is to become cytoplasmic messenger. These experiments suggest that this species of RNA has not been previously described.

The behavior of pulse-labeled RNA in the presence of actinomycin is presented in text-figure 6. These results show that the transformation of 45S RNA to 35S and 16S takes place in the presence of actinomycin. However, the subsequent behavior is abnormal. No 28S RNA reaches the cytoplasm in 2 hours, although previous data indicate that under normal

conditions considerable radioactivity would be expected in this cytoplasmic species of RNA. There has been considerable loss of radioactivity and optical density from the 30S peak in nuclear RNA by 2 hours. Since the previous results indicate that there is at most a small loss in radioactivity from the 35S RNA during its transition from a nuclear to a cytoplasmic stage, the breakdown in the presence of actinomycin is probably abnormal.

The results of the long label and chase experiment indicate that there is no high molecular weight RNA and in particular ribosomal RNA permanently associated with the nuclear preparation described. It is possible, of course, that ribosomes normally associated with the nucleus are lost during the preparation. The absence of radioactivity associated with 30S RNA in the nucleus from cells heavily labeled with tritiated uridine also indicates that there is little breakdown and reutilization of the labeled nucleotides from cytoplasmic ribosomes.

The experiments performed with the nucleolar fraction separated from the nucleoplasm indicate that the 45S RNA, a precursor to cytoplasmic ribosomal RNA, is all contained in the particular fraction that is designated "nucleolar." The 35S RNA also appears to be contained exclusively in this fraction. The nucleoplasm contains 28S RNA that labels after the nucleolar 35S but before the cytoplasmic 28S RNA. Thus the 30S peak in optical density visible in whole nuclear extracts is composed of the 35S nucleolar RNA and the 28S nucleoplasmic RNA. It should be emphasized that the sedimentation values used here are only approximate and have been chosen to conform with previous usage. It is unfortunate that sedimentation values have been used in the past as identifying nomenclature, especially since more careful experimentation indicates that the original assignments of S values are not exactly correct.

The experiments in which the separation of nucleoli was performed permit the following extension of the previous conclusions about the genesis of ribosomal RNA. The initial precursor of ribosomal RNA, the 45S RNA, is synthesized in the nucleolar fraction. After 25 minutes, the 45S RNA is cleaved to a 16S piece which is exported to the cytoplasm and a 35S piece which remains in the nucleolus. The 35S RNA is transformed into 28S RNA which is then found in the nucleoplasm. The data do not permit at this time a determination as to where this transformation takes place. After a stage in the nucleoplasm, the 28S RNA then emerges into the cytoplasm. It should be mentioned that the 28S nucleoplasmic RNA is probably in the form of a ribonucleoprotein particle.

The experiment on methylation was performed with a 10-minute pulse of a radioactive methyl donor. This length of time is presumably long compared to the synthesis of any of the species of RNA in the cell but relatively short compared to the time ribosomal RNA spends in any particular stage of development. In effect, a snapshot is obtained of the methylation process and, if extensive methylation of ribosomal RNA occurred at any time after synthesis, it could be observed as a peak in radioactivity

in the gradients shown in text-figure 9. Actually, the only significant peak in radioactivity is associated with the 45S RNA in the nucleolus. It appears that the methylation of ribosomal RNA takes place shortly after synthesis and no further methylation occurs. The absence of methylated RNA from the nucleoplasm indicates that the rapidly labeled polydisperse RNA is not methylated. It also indicates that the methylation of the 45S RNA in the nucleolus is probably true methylation and not due to the entry of label into the purine nucleotide pools since no radioactivity appears in the nucleoplasm where there is rapid incorporation of RNA precursors.

RESUMEN

Se describe un método para preparar una fracción nuclear de células HeLa. La fracción no contiene una cantidad apreciable del componente de ribosomas ARN 16S, y así parece estar libre de contaminación citoplásmica. Todos los precursores tempranos del ARN ribosómico citoplásmico están contenidos en la fracción nuclear.

El análisis de la sedimentación del ARN extraído de la fracción nuclear muestra un pico en densidad óptica de 45S y un pico mayor que sedimenta como 30S. Se muestra que el ARN en la región 30S es polidisperso, y contiene ARN 35S y 28S.

El trabajo previo ha demostrado que el evento inicial en la formación del ARN ribosómico es la síntesis del ARN 45S. Aquí se demuestra que el paso siguiente es la separación del ARN 45S para formar el ARN 16S, que emerge inmediatamente en el citoplasma, y el ARN 35S que permanece en el núcleo. El ARN 35S sufre una transición a 28S y entonces entra al citoplasma. La separación del 45S en ARN 35S y 16S se efectúa en presencia de actinomicina, pero la conducta subsiguiente del ARN 35S es anormal.

Experimentos en los que se persigue la marcación radioactiva muestran que no hay ARN de alto peso molecular permanentemente asociado con el núcleo.

Se describe un método para digerir la fracción nuclear usando un buffer con alta concentración iónica y DNasa. Se obtiene una fracción con partículas que morfológicamente semejan nucleolos y que tiene los precursores ribosómicos ARN 45S y 35S.

Un experimento preliminar sobre la metilación del ARN indica que el precursor ribosómico 45S es el único ARN de alto peso molecular que es metilado en un pulso corto.

REFERENCES

- (1) EAGLE, H.: Amino acid metabolism in mammalian cell cultures. *Science* 130: 432-437, 1959.
- (2) EARLE, W.: Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cells. *J Nat Cancer Inst* 4: 165-212, 1943.
- (3) SCHERRER, K., and DARNELL, J. E.: Sedimentation characteristics on rapidly labelled RNA from HeLa cells. *Biochem Biophys Res Commun* 7: 486-490, 1962.
- (4) GILBERT, W.: Polypeptide synthesis in *Escherichia coli*. II. The polypeptide chain and S-RNA. *J Molec Biol* 6: 389-403, 1963.
- (5) SCHERRER, K., LATHAM, H., and DARNELL, J. E.: Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 49: 240-248, 1963.

- (6) RAKE, A., and GRAHAM, A.: Kinetics of incorporation of uridine-C¹⁴ into L cell RNA. *Biophys J* 4: 267-284, 1964.
- (7) GIRARD, M., PENMAN, S., and DARNELL, J. E.: The effect of actinomycin on ribosome formation in HeLa cells. *Proc Nat Acad Sci USA* 51: 205-211, 1964.
- (8) PENMAN, S., SCHERRER, K., BECKER, Y., and DARNELL, J. E.: Polyribosomes in normal and poliovirus infected HeLa cells and their relationship to messenger RNA. *Proc Nat Acad Sci USA* 49: 654-662, 1963.
- (9) GIRARD, M., LATHAM, H., PENMAN, S., and DARNELL, J. E.: Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm. *J Molec Biol* 11: 187-201, 1965.
- (10) HOLTZMAN, E., SMITH, I., and PENMAN, S.: Electron microscopic studies of detergent treated HeLa cell nuclei. *J Molec Biol.* In press, 1966.
- (11) SWIFT, H.: Cytochemical studies on nuclear fine structure. *Exp Cell Res Suppl* 9: 54-67, 1963.
- (12) HYDE, B. B., SANKARANARAYANAN, K., and BIRNSTEIL, M. L.: Observations on fine structure in pea nucleoli *in situ* and isolated. *J Ultrastruct Res* 12: 652-667, 1965.

PLATE 55

With the exception of figure 7, all electron micrographs are from material fixed in glutaraldehyde, postfixed in osmic acid, embedded in Araldite, and stained with uranyl acetate and lead citrate. For details of preparation, discussion, and references, *see* (10).

FIGURE 1.—Intact HeLa cell. In the nucleus, *note* nucleoli (N) and the chromatin, part of which (*arrow*) is aggregated along the nuclear membrane. $\times 7,000$

FIGURE 2.—Cell swollen in RSB medium. The chromatin is more homogeneously distributed than in untreated cells. The nucleolus (N) is considerably less prominent except for the intranucleolar strands which stand out sharply. $\times 7,000$

FIGURE 3.—Portion of nucleus of untreated cell. Most of the field is occupied by part of a nucleolus. *Note* dense intranucleolar strands which sometimes are seen (*arrow*) at the border of regions of moderate electron density. Also present are numerous nucleolar granules (G) measuring approximately 10–20 $m\mu$ in diameter. For discussion and references concerning these components, *see* (10–12). $\times 25,000$

FIGURE 4.—Nucleus isolated from an RSB swollen cell by homogenization. Most of the cytoplasm is removed by this treatment, although up to 3% of the cells survive intact. The dense nucleolus is seen at N. In areas (*arrows*) the double nature of the nuclear envelope is visible. $\times 7,000$

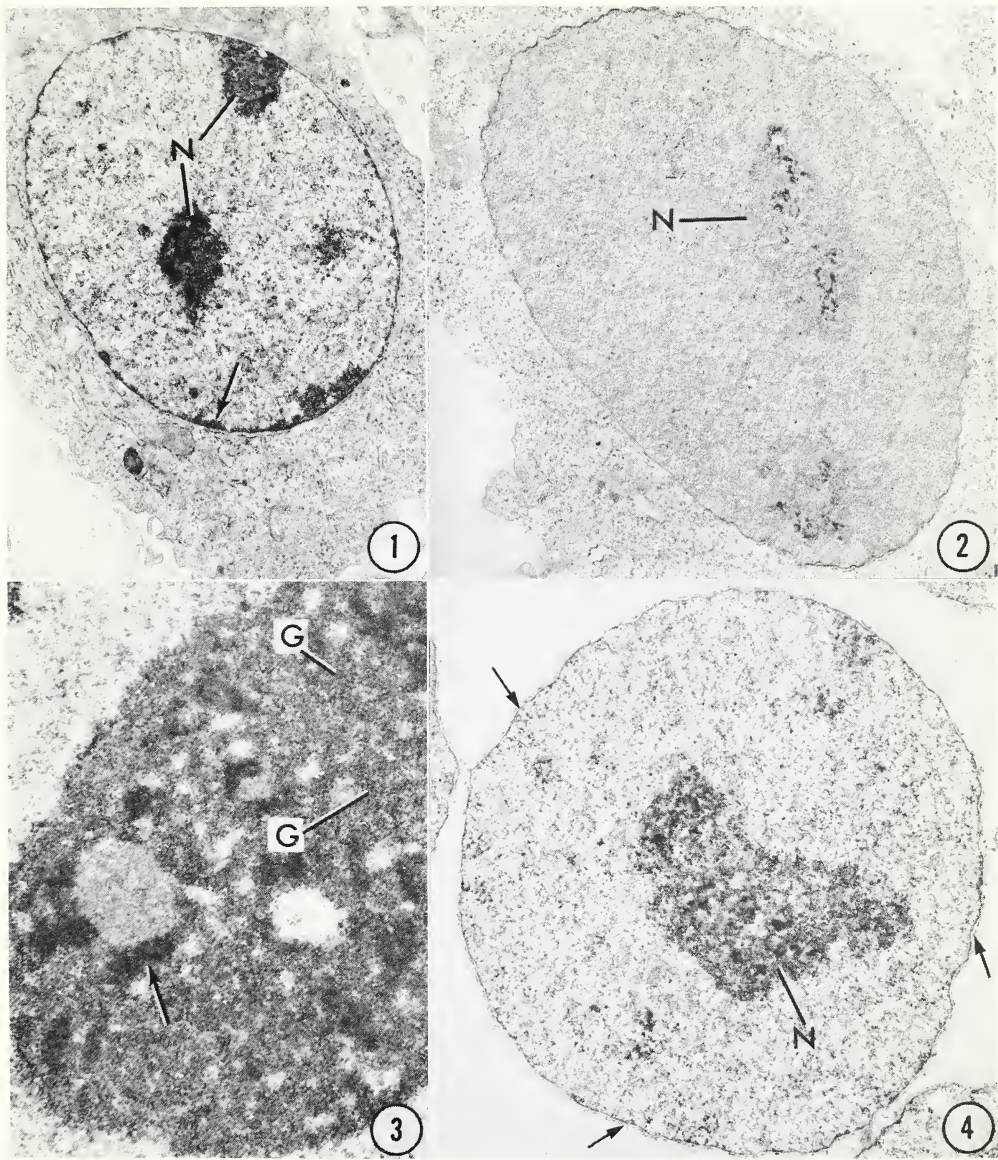


PLATE 56

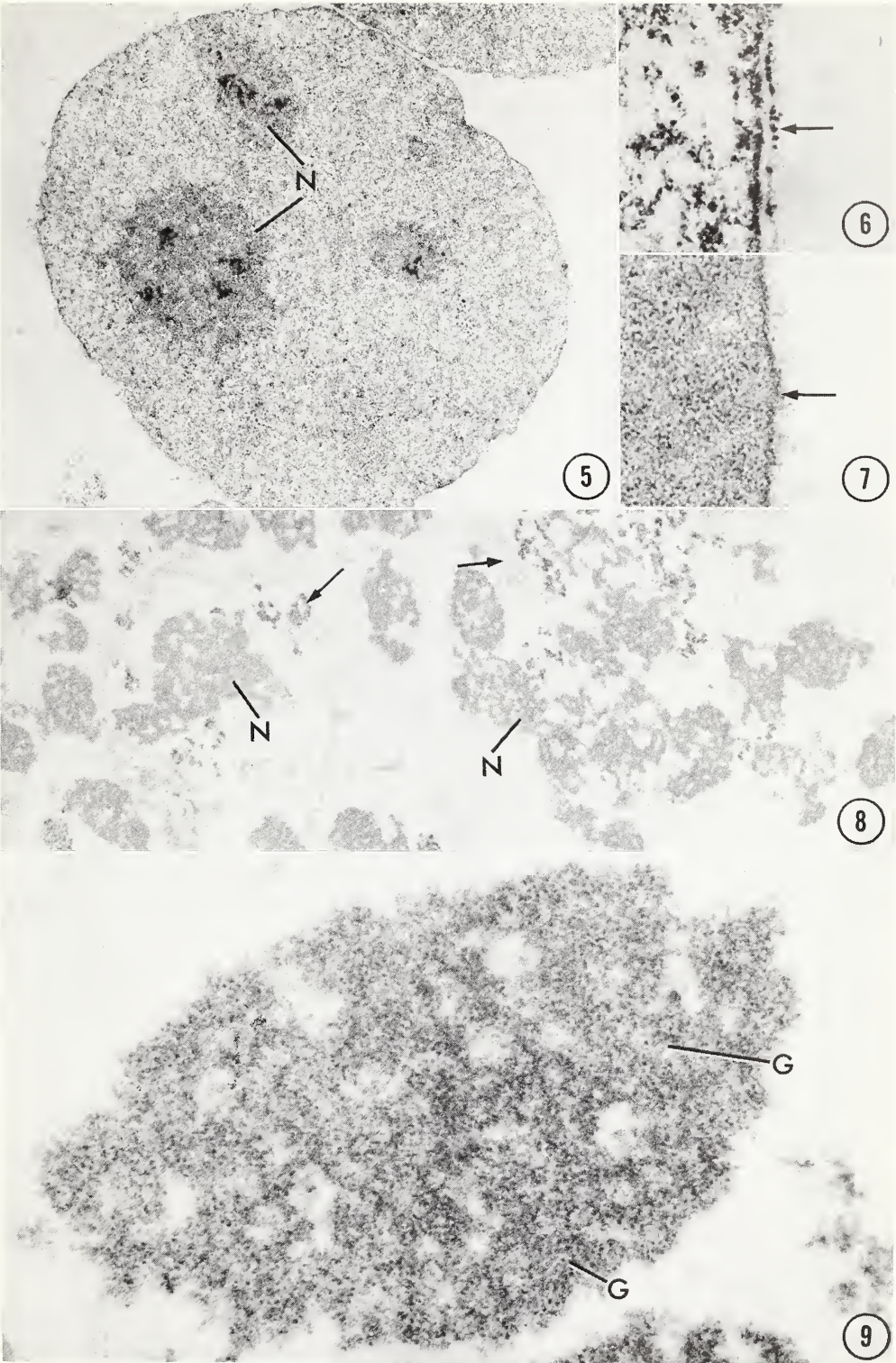
FIGURE 5.—Isolated nucleus after detergent treatment. The outer nuclear membrane is no longer visible (*cf* figs. 6 and 7). At N, the nucleoli may be seen to consist of dense intranucleolar strands surrounded by granules. Detergent treatment removes most cytoplasm left after homogenization; there are virtually no whole cells, and about 3% of the nuclei are appreciably contaminated, primarily with membranes derived, for example, from mitochondria. Biochemical data show that the nuclei contain no 16S RNA. Thus, the intranuclear granules surviving isolation and detergent treatment are apparently not mature ribosomes of the type found in the cytoplasm. $\times 9,000$

FIGURE 6.—Portion of nuclear envelope from nucleus similar to that shown in figure 4. Note the double nature of the envelope and the ribosome-like granules on the outer surface (*arrow*). $\times 47,000$

FIGURE 7.—Portion of surface of detergent-treated nucleus (*cf* fig. 5). This preparation was fixed in potassium permanganate and stained with lead citrate. The outer nuclear membrane has been removed by the detergent treatment. The membrane-like layer left at the surface of the nucleus (*arrow*) is not clearly a simple unit membrane; this may reflect changes due to the detergent. $\times 48,000$

FIGURE 8.—Portion of a pellet showing the effects of high salt and deoxyribonuclease on detergent-treated isolated nuclei. Much of the chromatin has been removed leaving partially purified nucleoli (N). However, considerable contamination by other nuclear components remains (*arrows*). $\times 8,000$

FIGURE 9.—Nucleolus from the same preparation shown in figure 8. The nucleoli are reasonably intact and continue to show the presence of many granules, some of which may be seen at G. $\times 34,000$



DISCUSSION

Feinendegen: On one slide you showed a sequential movement of label to the various peaks after actinomycin treatment. Wasn't there an indication that in the presence of actinomycin there is a continuous incorporation of the RNA precursor into DNA not different from the control?

Penman: Yes, that happens. Uridine is converted to cytidine, which in turn is converted to deoxycytidine and thymidine which are incorporated into DNA. These levels of actinomycin do not inhibit DNA synthesis. However, with short labeling periods there is no appreciable fraction of the uridine label in DNA. After about an hour, the amount of radioactivity in DNA derived from uridine starts to rise. For most of the gradients, but not all, we treated the preparation with deoxyribonuclease after alcohol precipitation. That usually removes the acid-precipitable radioactivity in the DNA region. In the most recent experiments, we have added deoxypyrimidines to the incubation mixtures in concentrations not high enough to be toxic, that is, to inhibit growth, but sufficiently high enough to partially suppress the incorporation of radioactivity into DNA. In the last slides, for instance, there is not very much radioactivity in the DNA regions because the experiments were performed in the presence of the deoxypyrimidines.

Siebert: I want to question the usefulness of the absence of 16S RNA from nuclei as a measure of purity. Do you have any cross check with a different method of preparing nuclei?

Penman: In a way, yes. Nuclear separation was sort of a hobby of mine for a year or so. I tried everything in the literature, including mechanical centrifuging through sucrose, and the preparations always had 16S RNA and also had obvious cytoplasmic contamination visible even in the light microscope. Now clearly, there could be cytoplasm without ribosomes; you could leach out the ribosomes and leave cytoplasmic tabs on the nuclei. But this could be true of any marker for cytoplasm, be it enzymatic, or anything else. With my present method, the electron microscope pictures indicate that in fact there are no structural entities corresponding to cytoplasmic contamination. So the electron microscope suggests that the absence of 16S RNA, and hence ribosomes, is not an unreasonable criterion. It is important to remember that in these experiments we do have a meaningful partition between nucleus and cytoplasm for RNA; we might not have a meaningful partition for protein.

The Nucleolus and Ribosomal RNA Synthesis in Mammalian Cells¹

G. P. GEORGIEV, *D. Biol. Sc., Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow*

SUMMARY

In this report the following questions are discussed: 1) some evidences of nucleolar origin of ribosomal RNAs; 2) the existence of two different precursors of ribosomal RNA and probably of two different pathways for 28S and 18S riboso-

mal RNA syntheses; 3) the structural organization of nuclear ribonucleoproteins: nucleolar nucleonemas and chromosomal 30S particles containing messenger RNA.—*Nat Cancer Inst Monogr* 23: 513–525, 1966.

THREE MAIN classes of RNA, ribosomal, transfer, and messenger, are synthesized in chromosomes and nucleoli of the animal cell. Many data indicate that one nucleolar function is synthesis of ribosomal RNA (rRNA). RNA formed during a short period of incubation of cells in the presence of radioactive precursor (so-called rapidly labeled RNA) is different from the rest of the cellular RNA with respect to sedimentation properties (1, 2). It is localized in chromosomes and nucleoli and consists of a mixture of RNAs with DNA-like base composition (dRNA) and rRNA-like base composition (3, 4). The former has been identified as newly formed messenger RNA of the cell (5, 6) and the latter as a precursor of ribosomal RNA (rRNA precursor) (7, 8).

To localize the site of rRNA precursor synthesis, three types of experiments were employed. In the first, pulse-labeled rat liver nuclei isolated in sucrose-glycerophosphate medium or Ehrlich ascites carcinoma nuclei isolated by osmotic shock were fractionated by salt solutions of different ionic strength. The base composition of RNA isolated from nuclear fractions obtained was determined (9, 10). The results from both tissues were identical. In the experiment shown in table 1, it can be seen that only a small amount of newly formed RNA was extracted after treatment with 0.14 M NaCl–0.001 M MgCl₂–0.01 M Tris, pH 7.0 (SMT, pH 7.0). This RNA was a mixture of rRNA and dRNA. As shown by the high G+C/A+U ratio, rRNA predominated. The rRNA of this fraction appears to

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

TABLE 1.—Distribution of newly formed RNA between fractions obtained by salt fractionation of isolated rat liver nuclei (P^{32} administered 1 hr before killing of animals)

Numbers of fraction	Solvent	Specific activity of RNA (cpm/mg RNA)	Percent of total activity of nuclear RNA	Base composition of newly formed RNA of fraction G+C/A+U
1.....	SMT pH 7.0.....	9, 200	6. 0	1. 30
2.....	SMT pH 7.8.....	37, 500	15. 2	0. 91
3.....	SMT pH 7.8.....	36, 800	14. 0	0. 78
4.....	SMT pH 7.8.....	45, 000	3. 5	0. 80
5 + 6.....	2.5 M NaCl + residue....	42, 000	61. 3	1. 31
6.....	Residue*.....	59, 000	30. 5	1. 65

*Residue fraction is that part insoluble in 2.5 M NaCl.

originate from nuclear sap ribosomes. Further extraction with SMT, pH 7.8–8.0, solubilized significant amounts of newly formed RNA, enriched with dRNA. The nuclear residue contained about two thirds of the total newly formed RNA, which from its G+C content must be predominantly rRNA and/or rRNA precursor. After the extraction of this with concentrated salt solution (2.5 M NaCl), the final residue contained relatively pure rRNA and/or rRNA precursor with very high specific activity. The same base composition was obtained whether it was determined by ultra-violet absorption or by P^{32} -radioactivity of RNA mononucleotides. When examined with the electron microscope, this residue consisted of nucleoli and chromosomal elements morphologically resembling nucleoli (11). Thus it appears that practically all newly formed rRNA is localized in nucleoli and partly in chromosomal nucleonemas.

In the second experiment the sensitivity of rRNA precursor and dRNA synthesis to low doses of actinomycin was compared (4, 10). In rat liver or Ehrlich ascites carcinoma cells treated by low doses of actinomycin (0.5 μ g per 1 ml of ascites or 50–100 μ g per rat), total RNA synthesis was inhibited by 50–60%. Under these conditions rRNA precursor synthesis was completely suppressed, whereas dRNA synthesis continued at the original rate. Autoradiographs indicate that nucleolar but not chromosomal RNA synthesis is blocked at this time (7, our data). Thus the nucleolus seems to be a site of rRNA precursor synthesis.

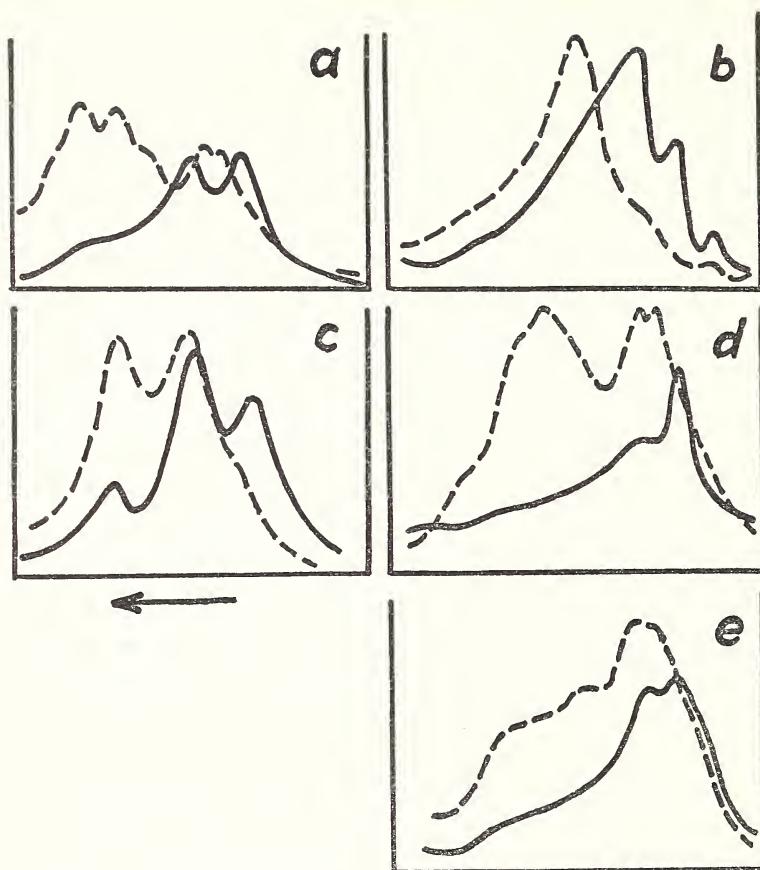
In the third experiment (preliminary data) hot phenol fractionation of cell nuclei was combined with autoradiographic analysis. Phenolic nuclei were isolated from Ehrlich ascites cells which had been incubated with H^3 -uridine for 30 minutes. The nucleoli, although deformed, were visible in nuclei obtained by treatment of cell suspension by phenol (pH 6 in the cold). These nucleoli contained labeled RNA. The subsequent treatments of such nuclear preparations with phenol at 40, 55, and 60 C caused the liberation of rRNA precursor, a mixture of precursor and dRNA, and pure dRNA, respectively (3, 4). After treatment at 40 C the

nucleoli were still visible in nuclei; after treatment at 55 C they had disappeared. Each successive phenol treatment caused a decrease in autoradiographic grain number per nucleus. The distribution of labeling between fractions, as determined by autoradiography and direct analysis, was the same. Only 15–20% of the control number of grains remained over nuclei after extraction at 63 C. It is significant that after treatment at 40 C, the labeled material was lost almost exclusively from nucleoli. Thus the suggestion that newly formed rRNA precursor is localized in nucleoli was further confirmed.

Although the results of these three experiments indicate that rRNA precursor synthesis proceeds in the nucleolus, we do not know whether all or only part of the precursor is synthesized there. As was pointed out earlier, the nucleolar preparations obtained by salt fractionation of isolated nuclei contained chromosomal threads also. We cannot exclude at the moment the chromosomal material as another site of rRNA precursor synthesis. Data concerning the distribution of DNA complementary to rRNA are also somewhat controversial. Ritossa and Spiegelman have shown this DNA to be localized in the nucleolar organizer region (12). According to other data, DNA complementary to rRNA is randomly distributed in the nucleus (13). For decisive conclusions, further experiments are needed and more convenient methods of isolation of nucleoli and nucleolus-associated chromatin must be elaborated.

PATHWAYS OF RIBOSOMAL RNA SYNTHESIS

Perry (7) and Scherrer, Latham, and Darnell (8) have shown by actinomycin-chase experiments that a heavy RNA is the precursor of rRNA. After pulse labeling, the cells were treated by actinomycin in doses sufficient to inhibit RNA synthesis completely. Under these conditions the heavy RNA disappeared and labeled peaks with sedimentation constants typical of rRNA appeared. However, these experiments did not supply information about the number of rRNA precursors and the relationship between different heavy RNAs and rRNA. To approach these questions more closely, we have investigated the sedimentation properties of heavy RNA fractions obtained by hot phenol fractionation. As was shown earlier (3, 14), 60–65 C treatment liberates the total RNA of nucleoli and chromosomes from preparations of “phenolic nuclei” obtained from the cells by treatment with phenol pH 6 in the cold. This RNA contains two poorly separated heavy peaks with sedimentation constants about 35–40S and 45S and labeled material in the 25–30S zone (text-fig. 1). Hot phenol fractionation resolves these components. Treatment at 40 C liberated only one heavy RNA fraction (35S) and two peaks of rRNA. After short pulses of radioactive precursor, the 40 C fractions contained only one labeled component: 35–40S RNA. This fraction is occasionally demonstrated by ultraviolet absorption as well as labeling procedures. At 55



TEXT-FIGURE 1.—Sedimentation diagrams of RNA fractions isolated from Ehrlich ascites carcinoma cells by the hot phenol method. (a) Total RNA of chromosomes + nucleoli (nucleolo-chromosomal RNA)—10–65 C fraction. (b–e) Fractions of nucleolar-chromosomal RNA obtained by hot phenol fractionation in temperature intervals: (b,c) 10–40 C; (d) 40–55 C; (e) 55–63 C. The cells were incubated with P^{32} for 1 hour (a, b, d, e) or 4 hours (c). Ultracentrifugation in 5–20% sucrose gradient, 25,000 rpm, 10–14 hours. Solid line—ultraviolet absorption; dotted line—radioactivity.

C another heavy RNA (45S) and also some 25–30S material were liberated. These fractions are resolved in sucrose gradients and can be investigated separately (text-fig. 1).

The following tests were used in the analysis of the nature of the fractions from the sedimentation experiments:

- 1) base composition of newly formed RNA;
- 2) the capacity for hybrid formation with homologous DNA;
- 3) competition with rRNA or with purified dRNA (63 C fraction) for complementary DNA strands.

One can see from table 2 that both heavy peaks, especially 35–40 S RNA, contain newly formed RNA with a high G+C content. Both hybridize

TABLE 2.—Characteristics of nuclear RNA fractions obtained from Ehrlich ascites carcinoma cells by combining hot phenol fractionation and ultracentrifugation in sucrose gradient

Time of incubation of cells with P ₈₂ (hr)	RNA fraction (interval of temperature treatment)	Sedimentation constant of peak in sucrose gradient	Base composition (G + C/A + U)	% of hybrid- ization (RNA/ DNA ratio = 1/40)	% of competition after addition of 5-10× RNA excess			
					dRNA (%)	rRNA (%)	28S rRNA (%)	18S rRNA (%)
1	10-40 C	35-40S	1.65	1.1	0	69	—	—
4	10-40 C	35-40S	1.60	0.95	0	75	67	53
4	10-40 C	28S	1.85	0.80	—	63	—	—
1	40-55 C	45S	1.40	1.1	0	65	—	—
1	40-55 C	25-30S	0.88	8.9	75	10	—	—
1	55-63 C	Total	0.81	11.2	70	0	—	—

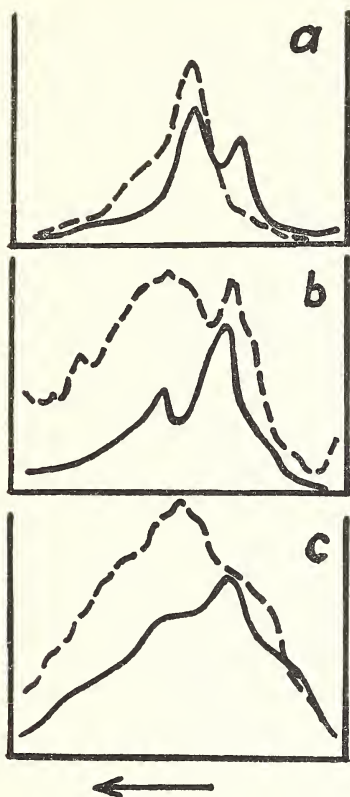
very poorly with DNA (about 0.5–1% of RNA added or 0.02% of DNA). The low hybridization capacity is typical of rRNA, whereas dRNA is much more active in this respect. Hybridization of heavy RNA with DNA was strongly inhibited by rRNA, but not by dRNA. On the other hand, the 25–30S component of the 55 C fraction contained newly formed RNA of high A+U type that effectively hybridizes with DNA and competes with dRNA but not with rRNA. Thus one can conclude that two heavy rRNAs exist in the cell, a 45S and a 35–40S. Both of these RNAs contain base sequences identical to that of rRNA. These data are in good agreement with the idea that these heavy RNAs are rRNA precursors.

Recently dRNA with a sedimentation constant of more than 35S has been demonstrated (15). In Ehrlich ascites carcinoma cells, the main part of newly formed dRNA is localized in the 25–30S zone and a relatively small part in the 45–50S zone (16, 17). Very probably, under some conditions, heavy dRNA peaks may be obtained, but this does not exclude the existence of two different RNA precursors of rRNAs. Recently Tsanev *et al.* isolated 3–4 heavy RNA peaks by using agar gel electrophoresis instead of gradient ultracentrifugation (18).

We have used long-term and actinomycin-chase experiments for analysis of relationships between heavy rRNA precursors and rRNA with sedimentation constants equal to 28S and 18S. In both experiments, RNA was fractionated by the hot phenol method. In this respect our experiments are different from Scherrer *et al.* (8). In our long-term experiments, the major labeling was observed in the 28S and 18S peaks. It is interesting that in the 40 C fraction the only rRNA labeled is 28S RNA, 18S labeling being completely absent. 28S RNA from the 40 C fraction had a base composition of the high G+C type and competed with rRNA during hybridization. It might be pointed out that base composition of both 35–40S and 28S RNAs of the 40 C fraction in long-term experiments corresponded, *i.e.*, they were richer in G+C than 18S rRNA (19). The same results with a 40 C fraction from rat liver were obtained by Venkov *et al.* (20). The analysis of the 55 C fraction is difficult because of the presence of dRNA in the 18S and 25–30S zones.

Actinomycin-chase experiments gave similar results (text-fig. 2). In Ehrlich ascites cells, actinomycin inhibited not only RNA synthesis but also the flow of labeled RNA from one fraction to another (21). Some part of the newly formed RNA is degraded, but part remains and its fate may be followed. In the 40 C fraction, 35S RNA disappeared and all the radioactivity of the fraction was found in the 28S peak. The 18S peak did not contain radioactivity. In the 55 C fraction, 45S RNA disappeared, and label was found in the 18S component. The latter contains dRNA as well as rRNA. However, in pure dRNA (65 C fraction) a labeled 18S peak was not present after actinomycin chase. It is very probable that the 55 C fraction contains labeled 18S rRNA.

One interpretation of the experiments described above is that there are two different polycistronic rRNA precursors—45S RNA for 18S rRNA



TEXT-FIGURE 2.—Sedimentation diagrams of RNA fractions isolated from Ehrlich ascites carcinoma cells after actinomycin chase. (a) 10–40 C fraction; (b) 40–55 C fraction; (c) 55–65 C fraction. *Solid line*—ultraviolet absorption; *dotted line*—radioactivity.

and 35–40S RNA for 28S rRNA. We have tried to verify this hypothesis by studying the competition between the rRNA precursors and two rRNAs for complementary sites in DNA (table 2). However, this method does not distinguish base sequences of the two rRNAs, since 28S and 18S components contain common sequences (21).

Let us point out once more that labeled RNA from 40 C fraction is localized in the nucleolus. Thus the synthesis of 35–40S RNA and formation of 28S rRNA proceed in the nucleolus of the animal cell. It is also possible that the synthesis of 45S RNA and formation of 18S rRNA are localized in the same place, but it cannot be excluded that these processes take place in the chromosomes.

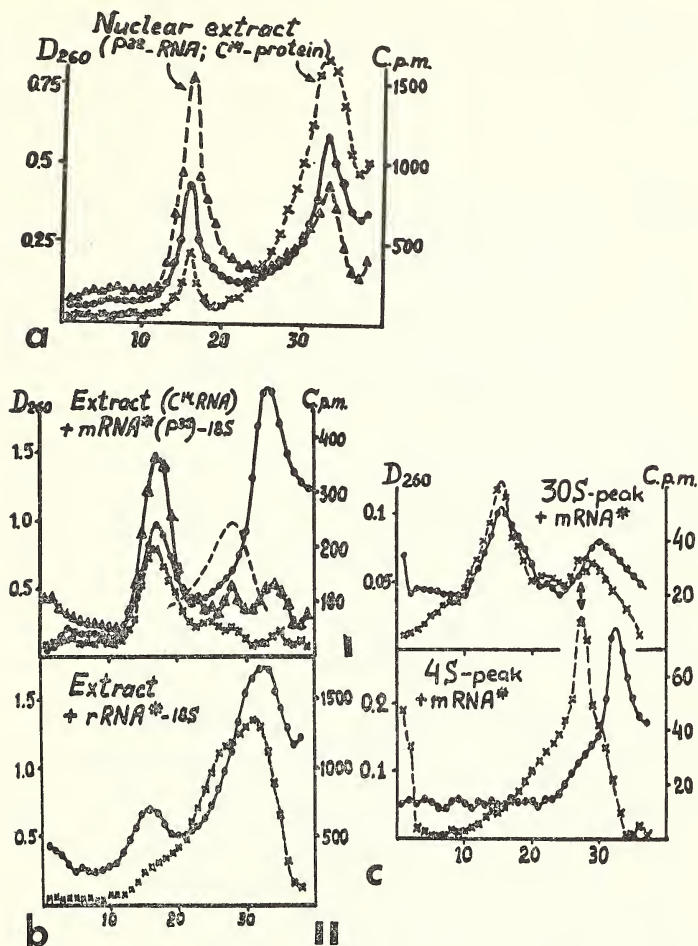
THE STRUCTURAL ORGANIZATION OF NUCLEAR RIBONUCLEOPROTEINS

The problem of structural organization of nucleolar ribonucleoproteins is of special interest because the nucleolus has often been suggested as the site of ribosome formation. The following fact is evidence in favor of this possibility. As was shown above, the cleavage of heavy rRNA pre-

cursor to rRNAs proceeds in nucleoli. Intensive protein synthesis also has been observed in nucleoli (22, 23). Ribosome-like particles have been isolated from nucleoli, although large and small subunits were not in a ribosomal-equivalent ratio (24). It is to be regretted that data concerning nucleolar ribosomes are so scanty.

We will discuss briefly some morphological properties of nucleolar ribonucleoproteins as observed in the electron microscope. As shown in table 1, newly formed rRNA precursor is present in the residue following extraction of cell nuclei with dilute and concentrated salt solution. In these residue fractions, the nucleolus appears as a network of fibrils 100 Å in diameter with 150 Å granules attached to them (fig. 1b). For this complex (a fibril with attached 150 Å granules) the term "nucleonema" was suggested (11). Nucleonemas are clearly visible in nucleoli before deoxy-nucleoprotein extraction, and for this reason we do not think they are artificial structures produced by 2.5 M NaCl treatment (fig. 1a). Depending on ionic strength, the nucleonemas are found to be separate from one another or gathered into relatively thick bundles 500–1000 Å in diameter (so-called nucleolonemas). The nucleonemal granules are sensitive to ribonuclease. As shown in table 1, they contain only rRNA and/or rRNA precursor, dRNA having been removed during dilute salt extraction. Thus one can conclude that nucleonemal granules are ribosomes or ribosomal precursors. So far, it has been impossible to localize the rRNA precursor in the nucleonemal complex because this RNA is only a small part of the total nucleolar RNA. It may be included in the fibrillar part of the nucleonemas, whereas the particles may be different stages of the pathway from the rRNA precursor synthesized on the DNA matrix to completed ribosomal subunits.

In conclusion we would like to consider the question of the nature of nuclear ribonucleoproteins containing dRNA that are solubilized by extractions with SMT, pH 7.8–8.0. In sucrose gradient ultracentrifugation of such extracts (15–30% sucrose, 12 hours with rotor SW25 at 25,000 rpm), the main part of newly formed dRNA was revealed as a highly homogeneous peak with sedimentation constant about 30S (text-fig. 3a). This component seems to be a ribonucleoprotein particle. Deproteinization of this extract by dodecyl sulfate sharply decreased the sedimentation constant of the newly formed dRNA (10–18S instead of 30S). With intact particles the labeled protein peaks coincide with the RNA peaks (text-fig. 3a). Thus a significant part of dRNA is contained in special homogeneous particles, with sedimentation constant about 30S (9). To understand the nature of 30S particles, the interaction of their dRNA and proteins was studied (25). When labeled, deproteinized dRNA was added to the nuclear extracts, the major portion of the RNA label appeared in 30S particles (text-fig. 3b). This reaction is specific for dRNA. When 18S rRNA- P^{32} was substituted for dRNA- P^{32} , no label appeared in the 30S peak (text-fig. 3b). To study the mechanism of this interaction, deproteinized dRNA was added to the 30S peak and to 4S proteins isolated by



TEXT-FIGURE 3.—Nuclear ribonucleoprotein particles containing messenger RNA (mRNA=dRNA), obtained from rat liver. (a) Sedimentogram of nuclear fraction extracted with SMT, pH 7.8. Extract contained RNA labeled with P^{32} and protein labeled with C^{14} . Solid line—Ultraviolet absorption; Δ —radioactivity (P^{32} RNA); X—radioactivity (C^{14} protein). (b) Interaction of nuclear extracts (containing RNA- C^{14}) and free RNA- P^{32} added to them. I—18S mRNA; II—18S rRNA. After the mixing of RNA with extract the material was ultracentrifuged in sucrose gradient. Solid line—ultraviolet absorption; Δ —radioactivity of proper RNA of extract (C^{14} RNA); X—radioactivity of RNA added (P^{32} RNA); dotted line—sedimentation curve of free RNA (obtained in separate experiment). (c) Interaction of 30S particles and 4S protein components of nuclear extracts with free 18S-mRNA- P^{32} . After mixing of peaks and mRNA the material was recentrifuged in sucrose gradient. Solid line—ultraviolet absorption; X—radioactivity. Arrows indicate the position of free mRNA.

sucrose gradient ultracentrifugation. After resedimentation of these mixtures, a labeled 30S peak occurred only in the first mixture [(dRNA + 30S peak) text-fig. 3c]. This and other experiments (25) provide evidence that dRNA binds to preexisting 30S particles, but not to proteins of lower molecular weight.

Only indirect data are now available concerning the biological role of 30S particles. Under conditions where RNA transport is blocked (actinomycin chase after pulse labeling), the 30S peak is absent or significantly reduced (25). Similar particles recently were found in the cytoplasm of animal cells (26). The kinetics of incorporation of labeled precursors into the dRNA of particles and that of endoplasmic reticulum is consistent with the existence of precursor-product relationships between these dRNA fractions (27). These facts indicate that 30S particles play a role in the transport of mRNA.

It has been suggested that 30S particles are the smaller ribosomal subunits (or their precursors) that interact with newly formed mRNA, removing it from the template-enzyme-product complexes on the chromosomes. The complexes of ribosomal subunits and mRNA thus formed are transferred to the cytoplasm where they bind to the larger ribosomal subunits. This hypothesis is consistent with the evidence that there are two different channels for the synthesis of rRNAs and of ribosomal subunits.

However, recent experiments, in which the direct analysis of base composition was made by measuring the ultraviolet absorption of separated 2'- and 3'-nucleotides, indicate that all the RNA in the 30S particles is dRNA. Its base composition coincides exactly with the base composition of the DNA. For this reason another interpretation of the nature of the 30S particles must be considered. Nuclear mRNA-protein complexes differ in some respects from informosomes, the mRNA-containing nucleoprotein particles found in the cytoplasm of embryos (28). For example, the homogeneity and buoyant density of these two types of particles are different. At present, it seems more likely that nuclear 30S particles are a special type of protein or lipoprotein particle which combines with mRNA and takes part in its transport. This, however, does not exclude that during the later stages of mRNA transport it may form a complex with the smaller ribosomal subunit.

In conclusion it should be stressed that there are many uncertainties in our knowledge about the structural organization and function of nuclear ribonucleoproteins. One of the reasons for these uncertainties is the absence of procedures which avoid RNA degradation during isolation of subnuclear components. The development of adequate procedures will facilitate progress in this direction.

RESUMEN

En este trabajo se discuten los siguientes problemas:

- 1) algunas evidencias del origen nucleolar de los ARNs ribosómicos;

- 2) la existencia de dos precursores diferentes del ARN ribosómico y probablemente de dos diferentes vías para la síntesis del ARN ribosómico 28S y 18S;
- 3) la organización estructural de las ribonucleoproteínas nucleares: nucleolonemas nucleolares y las partículas cromosómicas 30S que contienen el ARN mensajero.

REFERENCES

- (1) SCHERRER, K., and DARNELL, J. E.: Sedimentation characteristics of rapidly labeled RNA from HeLa cells. *Biochem Biophys Res Commun* 7: 486-490, 1962.
- (2) HIATT, H. II.: A rapidly labeled RNA in rat liver nuclei. *J Molec Biol* 5: 217-229, 1962.
- (3) GEORGIEV, G. P., and MANTIEVA, V. L.: Informational and ribosomal ribonucleic acids of nucleolochromosomal apparatus. Methods of separation and base composition. *Biokhimiia* 27: 949-957, 1962.
- (4) GEORGIEV, G. P., SAMARINA, O. P., LERMAN, M. I., SMIRNOV, M. N., and SEVERTZOV, A. N.: Biosynthesis of messenger and ribosomal ribonucleic acids in the nucleolochromosomal apparatus of animal cell. *Nature (London)* 200: 1291-1294, 1963.
- (5) SAMARINA, O. P., LERMAN, M. I., TUMANJAN, V. G., ANANIEVA, L. N., and GEORGIEV, G. P.: The properties of chromosomal informational ribonucleic acid. *Biokhimiia* 30: 880-893, 1965.
- (6) GEORGIEV, G. P.: Biosynthesis, transport and regulation of biosynthesis of informational RNA in animal cell. In *Molecular Biology. Problems and Perspectives* (Braunstein, A. E., ed.). Moscow, 1964, pp 109-116.
- (7) PERRY, R. P.: The cellular sites of synthesis of ribosomal and 4S RNA. *Proc Nat Acad Sci USA* 48: 2179-2186, 1962.
- (8) SCHERRER, K., LATHAM, H., and DARNELL, J. E.: Demonstration of an unstable RNA and of a precursor to ribosomal RNA in HeLa cells. *Proc Nat Acad Sci USA* 49: 240-248, 1963.
- (9) SAMARINA, O. P., ASRIJAN, I. S., and GEORGIEV, G. P.: The isolation of nuclear nucleoproteins containing messenger ribonucleic acid. *Dokl Akad Nauk SSSR* 163: 1510-1513, 1965.
- (10) LERMAN, M. I., MANTIEVA, V. L., and GEORGIEV, G. P.: Biosynthesis of ribosomal RNA in the nucleolus (nucleonemal apparatus of the cell). *Biokhimiia* 29: 518-528, 1964.
- (11) GEORGIEV, G. P., and CHENTSOV, J. S.: On the structure of the cell nucleus (electron microscopic studies of isolated cell nuclei). *Dokl Akad Nauk SSSR* 132: 199-202, 1960.
- (12) RITOSSA, F. M., and SPIEGELMAN, S.: Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc Nat Acad Sci USA* 53: 737-745, 1965.
- (13) CHIPCHASE, M. I. H., and BIRNSTIEL, M. L.: On the nature of nucleolar RNA. *Proc Nat Acad Sci USA* 50: 1101-1107, 1963.
- (14) GEORGIEV, G. P.: Ribonucleic acid of nucleolo-chromosomal apparatus. *Biokhimiia* 26: 1095-1107, 1961.
- (15) LERMAN, M. I., VLADIMIRTSEVA, E. A., TERSKICH, V. V., and GEORGIEV, G. P.: On the nature of newly formed RNA from animal cells. *Biokhimiia* 30: 375-387, 1965.
- (16) BRITTON, R. J.: Complementary strand association between nucleic acids and nucleic acid gels. *Science* 142: 963-965, 1963.
- (17) YOSHIKAWA, M., FUKADA, T., and KAWADE Y.: Separation of rapidly labelled RNA of animal cells into DNA-type and ribosomal RNA-type components. *Biochem Biophys Res Commun* 15: 22-26, 1964.

- (18) TSANEV, R. G., MARKOW, G. G., and DESSEV, G. N.: Incorporation of labelled precursors in the electrophoretic fractions of rat liver. *In* Symposium on The Methods for Estimation, Isolation and Fractionation of Nucleic Acids. Varna, 38, 1965.
- (19) MONTAGNIER, L., and BELLAMY, A.: 18S and 30S fractions from the RNA of Krebs-2 ascites cells with differing base composition. *Biochim Biophys Acta* 80: 157-160, 1964.
- (20) VENKOV, P. V., ANGELOV, E., and VALEVA, L. I.: Characterisation of thermally fractionated rat liver nuclear RNA. *In* Symposium on The Methods for Estimation, Isolation and Fractionation of Nucleic Acids. Varna, 32, 1965.
- (21) ATTARDI, G., HUANG, P. C., and KABAT, S.: Recognition of ribosomal RNA sites in DNA. II. The HeLa cell system. *Proc Nat Acad Sci USA* 54: 185-192, 1965.
- (22) SAMARINA, O. P.: The incorporation of labelled amino acids into protein fractions of nuclei from rat liver and ascites carcinoma cells. *Biokhimiia* 26: 61-69, 1961.
- (23) BUSCH, H., and STEELE, W. J.: Nuclear proteins of neoplastic cells. *Advances Cancer Res* 8: 41-120, 1964.
- (24) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosomes. *Biochim Biophys Acta* 76: 454-462, 1963.
- (25) SAMARINA, O. P., KRYCHEVSKAYA, A. A., and GEORGIEV, G. P.: Nuclear ribonucleoproteins containing messenger RNA. *Nature (London)* 210: 1319-1322, 1966.
- (26) HENSHAW, E. C., REVEL, M., and HIATT, H.: A cytoplasmic particle bearing messenger ribonucleic acid in rat liver. *J Molec Biol* 14: 241-256, 1965.
- (27) SAMARINA, O. P.: The observation of DNA-like RNA in the cytoplasm of animal cells. *Dokl Akad Nauk SSSR* 156: 1217-1220, 1964.
- (28) SPIRIN, A. S., BELITSINA, N. V., and AITCHOZIN, M. A.: Informational ribonucleic acids in early embryogenesis. *Z Obshtchei Biologii* 25: 321-338, 1964.

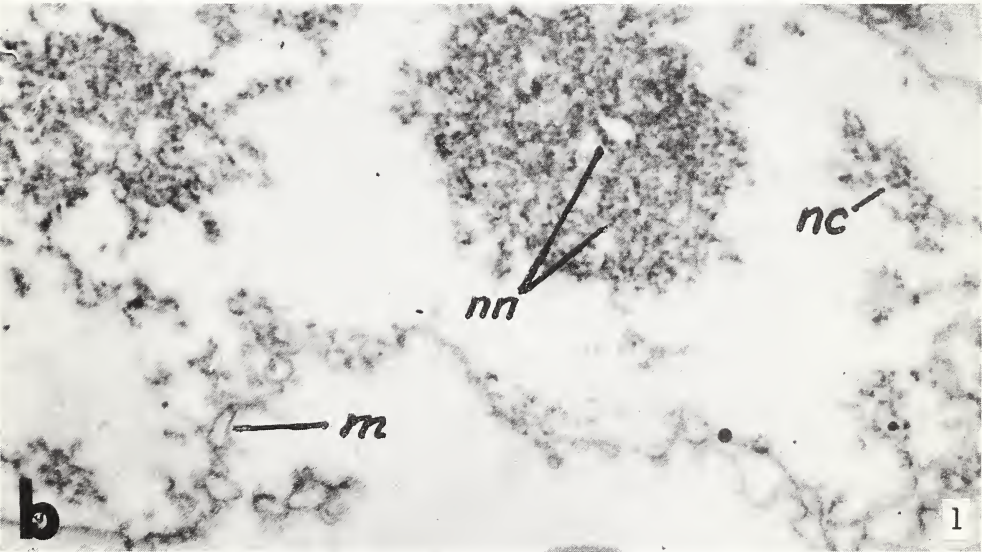
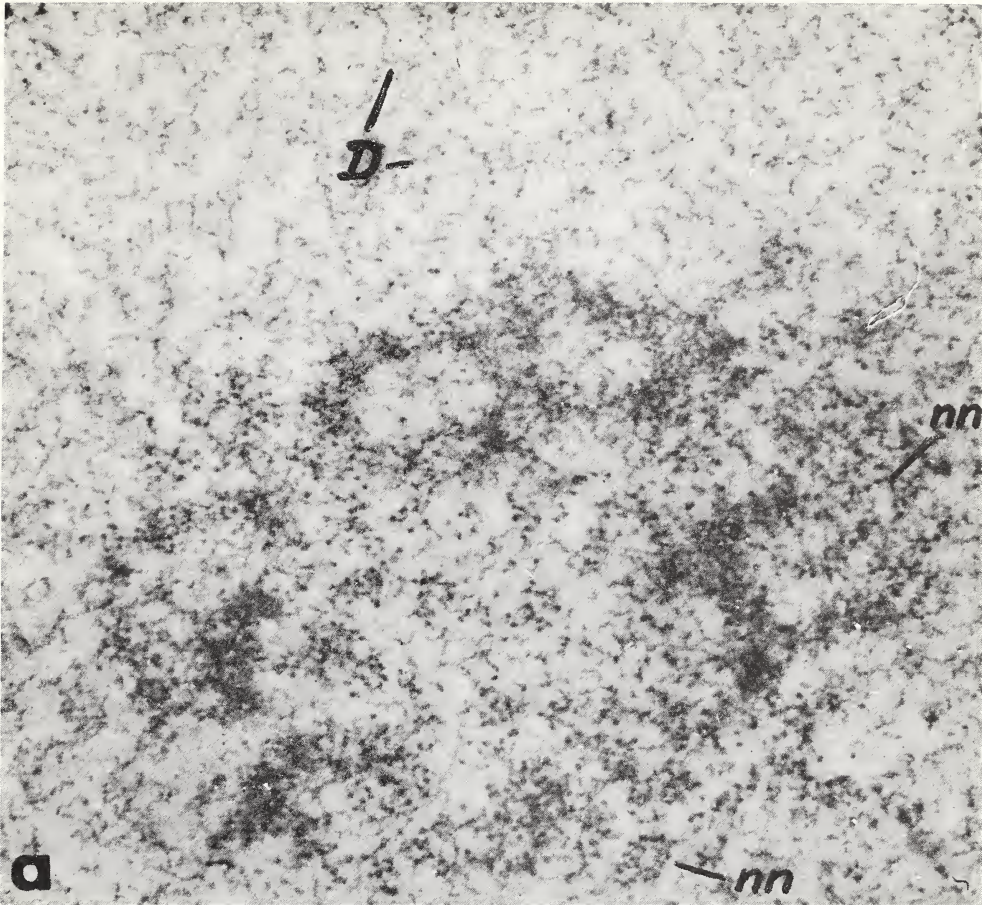
PLATE 57

FIGURE 1.—Electron micrographs of nucleolar preparations from isolated rat liver nuclei. $\times 40,000$

a) Nuclei extracted by 0.14 M NaCl, then placed into 0.1% potassium glycerophosphate-1 M sucrose medium fixed by 1% OsO₄ in the same medium, and stained by uranyl acetate.

b) Nuclei treated with deoxyribonuclease, extracted subsequently by 0.14 M NaCl and 2 M NaCl, and fixed by 1% OsO₄ in 2 M NaCl.

nn—Nucleolar nucleonemas; cn—chromosomal nucleonemas; D—DNP fibrils; m—membrane.



On Ribosome Biogenesis^{1, 2}

ROBERT P. PERRY, *Department of Molecular Biology,
The Institute for Cancer Research, Philadelphia, Pennsylvania 19111*

SUMMARY

One of the main metabolic functions associated with the nucleolus is the synthesis of ribosomal RNA and possibly the fabrication of ribosome particles. The bulk of available data is consistent with the following model. Cistrons coding for ribosomal RNA are transcribed into large 45S precursor molecules which are subsequently broken down within the nucleolus to 18S and 35S components. Then, by a mechanism which is still obscure, the RNAs are assembled together with protein into the ribosome subunits. The newly formed subunits appear in the cytoplasm as 40S and 60S particles, bearing 18S and 28S RNA, respectively. The

40S particles are rapidly transported to the cytoplasm, whereas the larger units dwell for a longer time in the nucleus, probably in the nucleolus. Strikingly, the newly synthesized 40S particles which appear in the cytoplasm are readily distinguishable from the bulk of the native 40S structures on the basis of their distinctive buoyant density. The significance of the mechanism of ribosome formation is discussed with reference to its possible role in the transport of information from nucleus to cytoplasm.—*Nat Cancer Inst Monogr* 23: 527–545, 1966.

I WOULD LIKE to state at the outset that I have come to this Conference with the conviction voiced by Dr. Brown that the nucleolus *is* the site of ribosomal RNA synthesis. Therefore, in my presentation I do not propose to discuss *whether* the nucleolus is involved in ribosome synthesis, but rather *how* and perhaps *why* it is involved. I shall begin with a brief summary of a model I presented last December at the previous Conference of this series in Buenos Aires. This model summarized the results of work on the nucleolus and the synthesis of ribosomes accomplished over the past 4 or 5 years. Although it was based largely on our own results with mammalian cell cultures, it was considerably influenced and supported by the work of some members at this Conference, such as Dr. Birnstiel, Dr. Pen-

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

² Supported in part by grant GB-4137 from the National Science Foundation and by Public Health Service research grant CA-06927 from the National Cancer Institute.

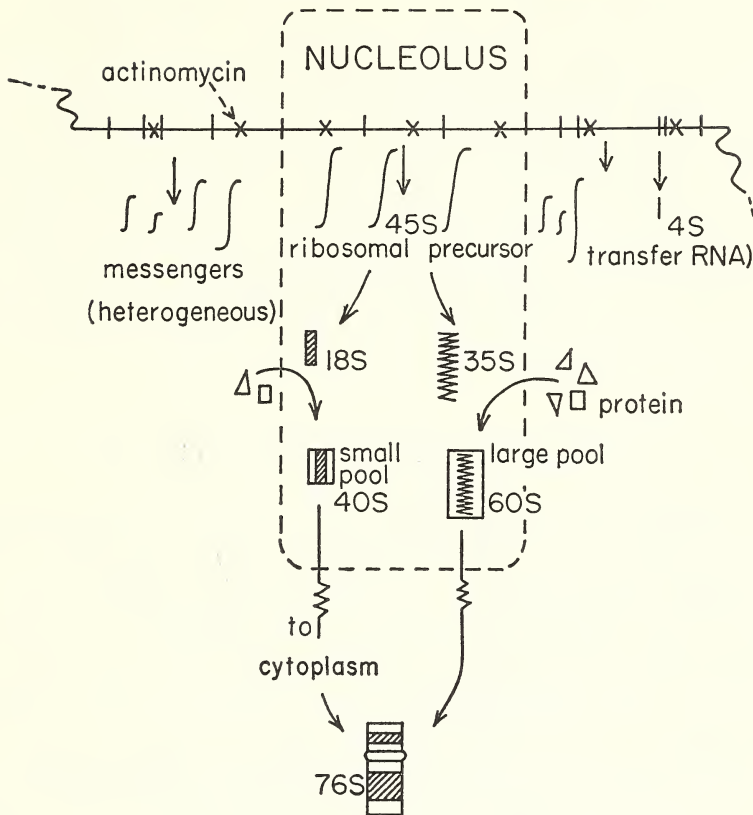
man, and Dr. Brown, and by the work of others unable to attend, such as Dr. Edström and Dr. McConkey [*see* (1) for references].

After discussing the model, I will consider in detail some particular characteristics of ribosome particles and their subunits which are germane to an understanding of their biogenesis. Strictly speaking, much of the material to be presented here is not directly concerned with the nucleolus. For the most part we will be examining the particles as they appear in the cytoplasm subsequent to nucleolar involvement. Nevertheless, I believe a more detailed knowledge of this stage of ribosome biogenesis may ultimately help us gain a more profound understanding of the role of the nucleolus in the process. In this connection I want to introduce another question which has come up in the last year or so, namely, whether in cells of higher organisms a ribosome subunit may serve as the vehicle for the transport of messenger RNA from the nucleus to the cytoplasm. Although I do not have a definitive answer, I wish to introduce the topic here since it has not been mentioned at this Conference. Perhaps we can discuss it further in the Panel and the open Discussion that follows.

MODEL OF NUCLEOLAR INVOLVEMENT IN RIBOSOME FORMATION

Text-figure 1 schematically shows a segment of genome containing the nucleolar region (marked off by the dotted line). Whereas the nucleolar genes produce the 45S molecules that are the precursors of ribosomal RNA (rRNA), other regions of the genome are responsible for the transcription of 4S transfer RNA (tRNA) and of heterogeneous messenger components (mRNA) ranging in size from greater than 45S down to about 6S. Our evidence supporting these transcriptive aspects of the model comes mainly from studies with actinomycin D, used in low concentrations to inhibit specifically the synthesis of the 45S component, and, equivalently, as seen by autoradiography, the synthesis of nucleolar RNA. The residual synthesis of RNA, which occurs almost exclusively in the chromatin region of the nucleus, was analyzed with respect to several properties, including sedimentation, base composition, and complementarity to DNA, to give the scheme shown here (1, 2). There is some controversy as to whether the tRNA cistrons are located close to the nucleolus, as suggested by the experiments of Birnstiel and Sirlin (3, 4), or remote from the nucleolus, as proposed by Woods (5, 6) and Ritossa (7). Our earlier experiments also indicated a separation of the sites of synthesis of ribosomal and 4S RNA, and so I tend to favor the latter view. However, at the moment, the possibilities exist that a relatively small part of the tRNA may be transcribed in the nucleolar region or that the proximity of rRNA and tRNA cistrons might vary from system to system.

Next, let us consider the transformation of the 45S precursor into the relatively stable 18S and 28S components which exist in the cytoplasmic



TEXT-FIGURE 1.—Model of RNA synthesis and nucleolar function. For description, see text. Other details in (1).

ribosomes. For elucidation of an early stage of the process, advantage was taken of the fact that a purine analogue, 8-azaguanine, can apparently block a step after the initial breakdown of the 45S component into 35 and 18S RNA. By comparing autoradiographs with sedimentation analyses of radioactively labeled RNA, we showed that the conversion of 45S to 18S and 35S occurs before the newly formed RNA leaves the nucleolus (1, 8). The 35S component is ultimately converted to 28S, but the nature of this transition and the site in the cell where it occurs are not completely clear (*see Panel Discussion*). To study the later stages in the transformation, we focused our attention on the cytoplasm and observed both the RNAs and the ribonucleoprotein particles as they emerged from the nucleus. One of the most striking things we found (1), and this was also noted by Dr. Penman and his collaborators (9, 10), was that after brief pulses with labeled nucleosides, 40S particles containing labeled 18S RNA appear in the cytoplasm considerably in advance of labeled 60S particles and labeled 76S monoribosomes. Since we believed that the labeled 40S and 60S particles represented newly formed ribosome subunits, we antic-

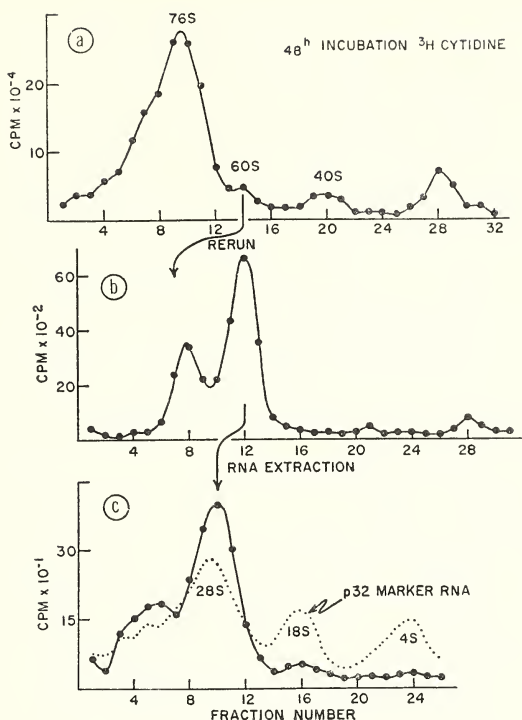
THE NUCLEOLUS

ipated a strict stoichiometry, that is, every 40S particle which leaves the nucleus should be accompanied by a 60S particle. Therefore, in order to explain the labeling data, we proposed that the nucleolus contains a large pool of 60S particles and a small pool of 40S particles, so that as a result of dilution in the respective pool, the passage of radioactivity would be retarded to a greater extent for the 60S than for the 40S particles (1). Of course, considerable credence to this idea was given by Dr. Birnstiel's observation that pea nucleoli contain a high proportion of the large subunit in comparison to the cytoplasmic ribosomes (11). Our recent data have confirmed and extended this idea. The difference in pool size could be related ultimately to a differential rate of fabrication of completed particles, as suggested by Girard *et al.* (10).

ANALYSIS OF CYTOPLASMIC PARTICLES

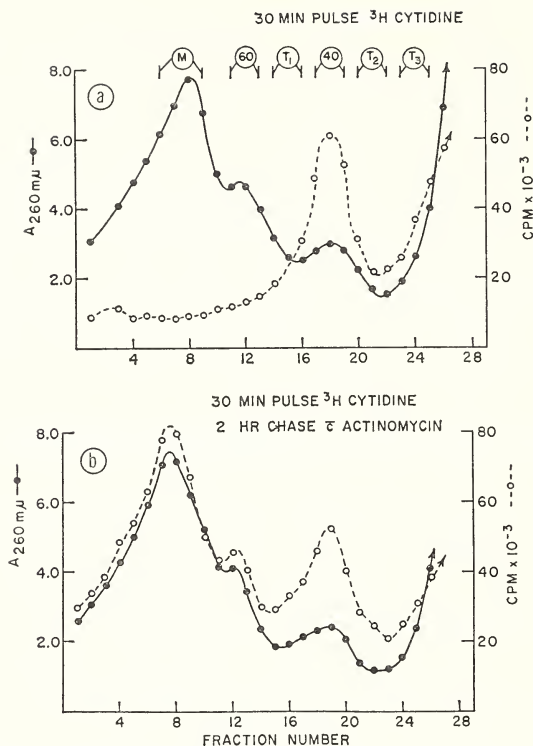
As an example of the general kind of analysis made on the cytoplasmic particles and the problems encountered, let us consider the determination of the type of RNA component contained in the 60S particle. After homogenization of about 0.5 g wet weight of L strain cells harvested from a suspension culture labeled for two generations in H^3 -cytidine, a $16,000 \times g$ supernatant fraction was prepared and layered onto a 15–30% sucrose gradient (1). The radioactive profile observed after 12 hours centrifugations at $60,000 \times g$ is shown in text-figure 2a. In addition to a large 76S monoribosome peak, small peaks of native 60S and 40S particles are obtained. Several factors influence the relative amounts of these 3 components: the concentration of magnesium present, whether nonionic detergents or deoxycholate is used in the preparative procedure, and the metabolism of the cells themselves. This particular sample of cells had almost reached saturation phase, and the amount of monoribosomes far exceeded the amount of the native 40S and 60S RNP structures in the cytoplasm. On the other hand, cells in the exponential growth phase contain a larger proportion of the 40S and 60S particles [text-fig. 3, also (12)].

To show that a particular RNP particle contains a particular component of RNA, it is necessary to obtain rather pure preparations of each particle. This is not too difficult for monoribosomes and 40S particles, but for the 60S particles a second zonal centrifugation is usually required. Text-figure 2b, showing a rerun of the 60S peak fraction, illustrates the amount of contamination one can expect in such initial sucrose gradients. This should serve as a cautionary note for analyses of peaks on the basis of a single zonal centrifugation. When RNA is extracted from the rerun 60S particles, one obtains predominantly the 28S component (text-fig. 2c). The small peak at about 35S is probably a sedimentation artifact, since the marker RNA also showed some spreading in this region. The important point here is that the amount of 18S RNA in the 60S particle is essentially negligible. All that is found can be explained by persistent contamina-



TEXT-FIGURE 2.—A suspension culture of L cells, 800 ml, initially containing 1.5×10^5 cells/ml was incubated for 48 hours with $0.5 \mu\text{C}/\text{ml}$ of H^3 -cytidine. Final cell concentration was approximately 6×10^5 cells/ml. After harvesting, the cells were swollen in approximately 2 ml hypotonic buffer and homogenized. The homogenate was centrifuged at $16,000 \times g$ and the supernatant layered onto a 27 ml 15–30% gradient of sucrose in 0.01 M Tris buffer, pH 7.8, containing 0.05 M NH_4Cl and 0.005 M Mg^{++} and centrifuged for 10 hours at 25,000 rpm. Aliquots of each fraction were precipitated in 5% trichloroacetic acid (TCA) and counted. Other details of above procedure in (1). Fraction number 14, corresponding to the 60S peak was effectively dialyzed by passage through a small column of Sephadex G25 and rerun as above on a 27 ml 15–30% sucrose gradient. Fraction number 12 of the rerun, corresponding to the purified 60S peak, was diluted with 2 volumes of 0.01 M acetate buffer, pH 6.0, containing 0.1 M NaCl and 10^{-3} M Mg^{++} , and extracted with 1% sodium dodecyl sulfate and phenol at 25 C. The RNA was precipitated in 70% ethanol together with about 350 μg of P^{32} -labeled ribosomal and 4S RNA which served as a marker, redissolved, layered onto a 4.5 ml 5–20% sucrose gradient, and centrifuged for 3 hours at 39,000 rpm. (a): Radioactivity profile of initial zonal gradient. (b): Radioactivity profile of rerun gradient. (c): Solid curve: H^3 -labeled RNA from 60S particles; dotted curve: P^{32} -labeled marker RNA.

tion from the monoribosomes. As did Dr. Penman and his co-workers (10), we interpreted our earlier experiments to mean that the 60S particles contain both 28S and 18S RNA components. But now it is quite obvious that the 40S and 60S particles contain solely 18S and 28S RNA, respectively [*cf.* also (13)].



TEXT-FIGURE 3.—Zonal sedimentation diagrams of $16,000 \times g$ supernatant fractions prepared and analyzed as in text-figure 2. Sixteen hundred ml of L cell culture (4.4×10^5 cells/ml) was concentrated eightfold and incubated for 30 minutes with $5 \mu\text{g/ml}$ H^3 -cytidine. One half of the culture was harvested (a); the other half was diluted to 800 ml and incubated for 2 hours with 0.05 mg/ml each of unlabeled cytidine and uridine and $0.4 \mu\text{g/ml}$ of actinomycin D (b). Solid curves: absorbance at $260 \text{ m}\mu$. Dotted curves: radioactivity. The encircled symbols in (a) refer to typical fractions used for the buoyant density studies described later.

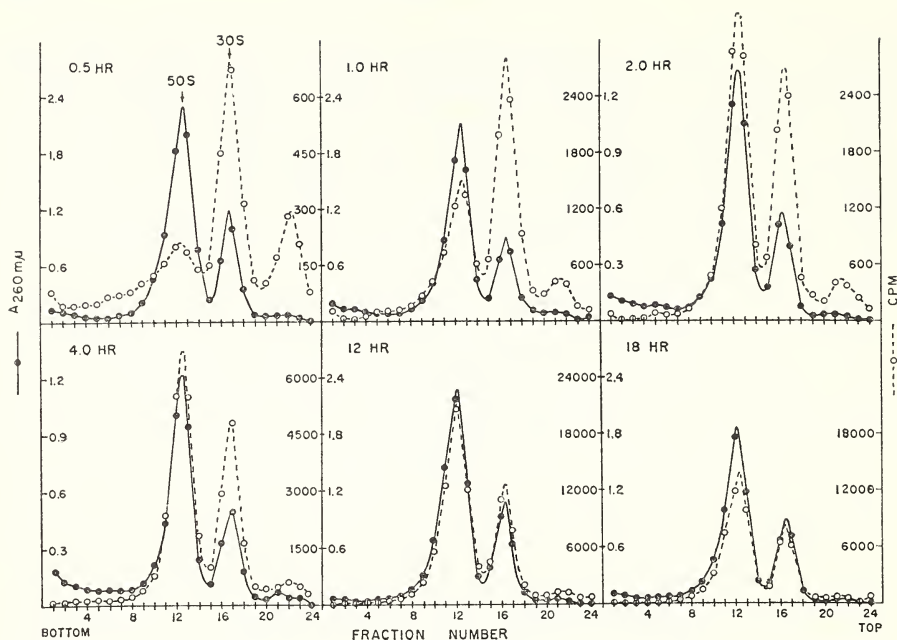
We have investigated the rapid appearance in the cytoplasm of radioactively labeled 40S structures and the much slower appearance of radioactivity in the 60S particles. This phenomenon is illustrated in text-figure 3 which shows a pair of profiles of cytoplasmic particles from a recent pulse-chase type of experiment. After a 30-minute pulse with H^3 -cytidine there was negligible radioactivity in the monoribosomes and the 60S particles compared to that in the 40S region. But after two hours' incubation, even in the presence of a relatively high concentration of actinomycin D ($0.4 \mu\text{g/ml}$), appreciable radioactivity appeared in the heavier particles. If the incubation is continued further, the 40S radioactivity decreases until there is nearly uniform specific activity in all particles. It is interesting that one can obtain this result in the presence of actinomycin because, as indicated by Dr. Penman, there is some evidence that actinomycin can block the migration of rRNA from the nucleus to

the cytoplasm (9). However, we have not found this to be the case when pulse times were of the order of 30 minutes. If the pulse is shortened to 10 minutes, chasing with actinomycin still leads to a labeling of monoribosomes and 60S particles, but the yield is less than one would expect on the basis of the amount of 45S RNA synthesized during the pulse [*cf.* also RNA studies (1, 8)]. The rRNA precursor components in the nucleus are apparently stabilized after 30 minutes, so that they are relatively insensitive to the actinomycin. Perhaps this corresponds to conversion of the 45S component to 35S and 18S.

From the work of Girard and co-workers (10), it appears that the flow of radioactive 18S RNA proceeds from the 40S particles to the polyribosomes to the monoribosomes. If, as previously proposed, the early labeled 40S particles are accompanied by unlabeled 60S particles, and, in addition, if these particles become ribosome subunits *and remain together after release from the polyribosomes*, then one should find that the early labeled monoribosomes consist of labeled 30S subunits bound to unlabeled 50S subunits.³ To test this idea we isolated particles from groups of cells which had been first pulsed with H³-uridine for 30 minutes and then chased with unlabeled nucleosides for various periods. Monoribosomes taken from the 76S peaks were dissociated by chelating the magnesium with ethylenediaminetetraacetate (EDTA), and the kinetics of labeling of the 50S and 30S subunits were measured (text-fig. 4). After chasing for 30 minutes, the 30S subunits of the monoribosomes are clearly labeled, but there is little radioactivity associated with the 50S structures. This difference in labeling, which is still marked at 60 minutes, gradually disappears upon further chasing. Extraction and analysis of the RNA from the monoribosomes demonstrated that the label in the 30S and 50S particles was in 18S and 28S RNA, respectively. The specific activities of the derived 30S and 50S particles, calculated from the data in text-figure 4, are shown in text-figure 5. After an initial lag of about 30 minutes, the 50S specific activity increases at a slightly slower rate than the 30S specific activity. The maximum specific activity of the 30S subunit is greater than that of the 50S subunit. This may be due in part to the fact that the 18S rRNA contains 20–25% more uracil than the 28S rRNA (14). Finally, after extended chasing, when the intracellular pools of nondiffusible radioactivity have been exhausted, there is a decay in specific activity of both particles.

Thus we see that soon after labeling, some of the ribosomes which are in the cytoplasm consist of a small labeled subunit and a large unlabeled subunit, a fact consistent with the suggestion that these structures are released

³ The sedimentation coefficients of the ribosome subunits are 30S and 50S when the particles are derived by dissociation of monoribosomes in the presence of chelating agents such as EDTA. On the other hand, when these derived subunits are mixed with the 16,000 $\times g$ cytoplasmic supernatant and sedimented in a 15–30% sucrose gradient, they sediment at approximately 40S and 60S (JV). The cause of this difference is not definitely known although recent measurements of the buoyant densities of these particles have led us to suspect that the 30S and 50S particles are compact structural variants of the 40S and 60S structures, respectively, rather than particles containing less material.

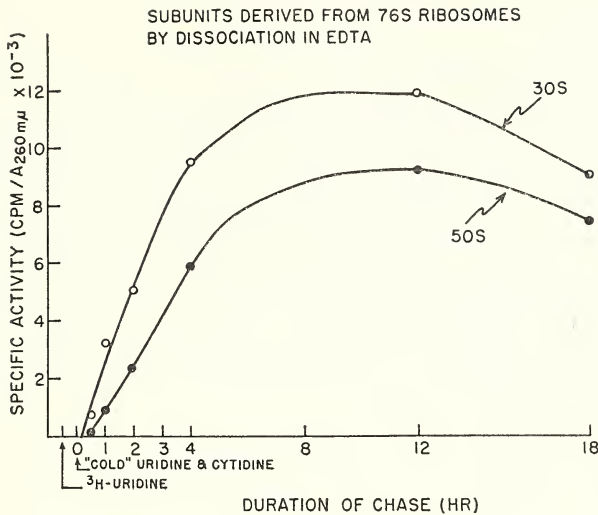


TEXT-FIGURE 4.—Fifteen hundred ml of cells containing 4.3×10^5 cells/ml were concentrated sevenfold and incubated for 30 minutes with $0.5 \mu\text{C}/\text{ml}$ H^3 -uridine. The cells were then diluted to 1500 ml and chased with 0.05 mg/ml each of unlabeled uridine and cytidine for 0.5, 1.0, 2.0, 4.0, 12, and 18 hours. From each sample, $16,000 \times g$ supernatant fractions were prepared and subjected to zonal centrifugation as in text-figure 2. For each time point the fractions corresponding to the monoribosome peaks were pooled, diluted in Tris- NH_4Cl buffer, and pelleted by centrifugation at 50,000 rpm for 2 hours. The pellets were resuspended in the same buffer containing 10^{-3} M EDTA and centrifuged for 3 hours at 39,000 rpm in 15–30% sucrose gradient containing 10^{-3} M EDTA. Solid curves: absorbance at 260 μm . Dotted curves: radioactivity. The inequality in early labeling of the derived particles indicates the presence of monoribosomes containing a 30S subunit which is more recently synthesized than its companion 50S subunit.

from the polysomes without dissociation. Previously it was demonstrated that the major portion of the rapidly labeled cytoplasmic RNA consists of an 18S component (1, 15). That this RNA is associated with the 30S subunit of a monoribosome further supports the hypothesis that much of the rapidly labeled RNA demonstrable earlier in the 40S particles consists of ribosomal rather than messenger components (*vide infra*). Using cells from rat liver, Hadjiolov (16) has performed a similar experiment with essentially the same result, although in his case the source of the subunits necessarily included the polyribosomes.

BUOYANT DENSITY STUDIES

As a next step in the analysis of the cytoplasmic particles, we made measurements of buoyant density, another useful parameter for resolving RNP



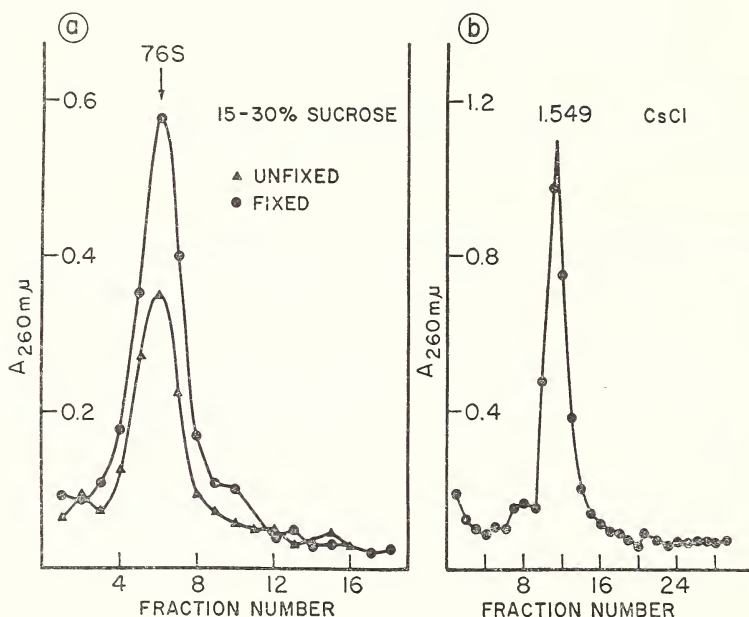
TEXT-FIGURE 5.—Specific activities, calculated from the integrated peaks of absorbance and radioactivity in text-figure 4, *versus* duration of chase.

particles of differing properties (17). We used the method of Spirin (18), in which cesium chloride banding is achieved without degradation of the particles by first cross-linking the RNA and protein with formaldehyde. Very reproducible, sharp banding patterns in cesium chloride density gradients were obtained from RNP particles purified by zonal centrifugation on sucrose gradients and fixed immediately in formaldehyde, thus giving us a new dimension for analysis of the properties of these structures.

Actually, the *raison d'être* for the buoyant density studies was to investigate the nature of the rapidly labeled 40S particles. It had been proposed that an appreciable portion of radioactive RNA, though appearing to sediment like pure 18S ribosomal species, may in fact represent a message component (12, 19). Accordingly, in a cell system such as ours, the rapidly labeled RNA of the 40S particle might consist of a mixture of newly formed 18S rRNA and mRNA. A weakness of this hypothesis is that it is difficult to conceive how such an mRNA-40S particle complex could sediment as it does, at the same rate as the bulk of the 40S particles. It might also be predicted that if some of the label represents additional RNA in the form of attached message, then the rapidly labeled 40S particles would have a significantly higher proportion of RNA to protein, and consequently a greater buoyant density, than the bulk of the 40S particles. On the other hand, one might consider an alternative hypothesis, such as the existence of mRNA-protein complexes which sediment in the same region of a sucrose gradient as the 40S particles (20-22). Such structures would not necessarily have a density greater than the 40S particles and might even have a lower density. There is a possibility,

then, that buoyant density studies can help decide between these two hypotheses (17).

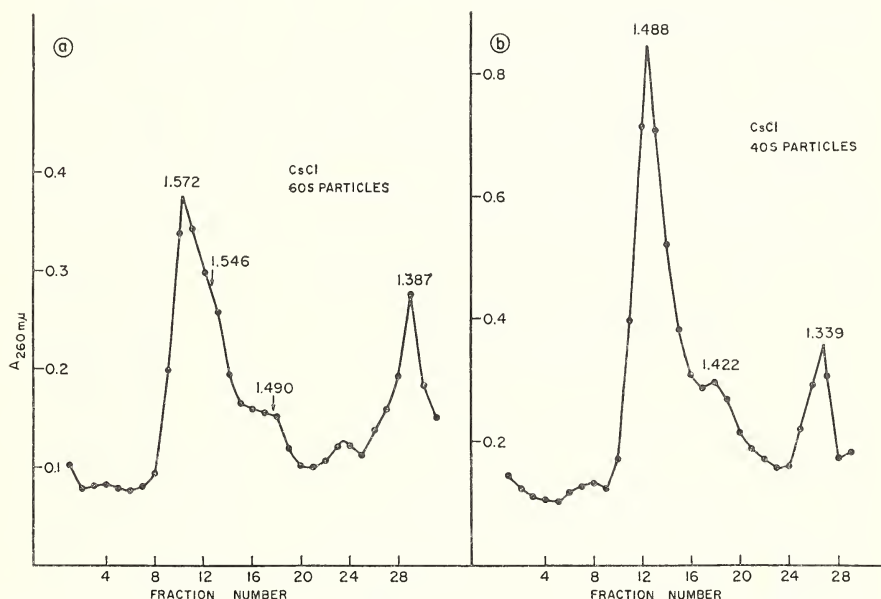
For a valid interpretation of the results of these studies, it is necessary to know whether the formaldehyde reaction alters the sedimentation characteristics of the particle. Text-figure 6a illustrates comparative sedimentation behavior of fixed and unfixed monoribosomes and shows that the cross-linking has no measurable effect on the sedimentation constant of the particle. Similar data have been obtained with smaller particles. When the fixed monoribosomes (text-fig. 6a) were banded to equilibrium in a cesium chloride gradient that ranged in density (ρ) from 1.7–1.3 g/cm³, we observed the very sharp band at $\rho = 1.549$, seen in text-figure 6b. The average buoyant density of the monoribosome, calculated from many determinations, was $\rho = 1.546$.



TEXT-FIGURE 6.—Monoribosomes were isolated from sucrose gradients prepared as described in text-figure 2 except that the sucrose was dissolved in a buffer containing triethanolamine-KCl (TEA) rather than Tris-NH₄Cl (17). Part of the sample was fixed in 6% formaldehyde (HCHO) for 24 hours at 5 C and then dialyzed against TEA buffer (17). (a) Recentrifugation of fixed particles (●) on 15–30% sucrose gradient (3 hours, 39,000 rpm) compared to a companion run with unfixed particles (▲). (b) Equilibrium sedimentation (46 hours at 35,000 rpm) in a CsCl density gradient ($\bar{\rho} = 1.54$ g/cm³) of the fixed monoribosome preparation shown in (a).

When the 40S and 60S particles were banded on CsCl, somewhat more complex patterns resulted (text-fig. 7). With the 60S particle (text-fig. 7a) the predominant band is at about $\rho = 1.57$, 0.02–0.03 units higher than that observed for the monoribosome. This particular preparation was

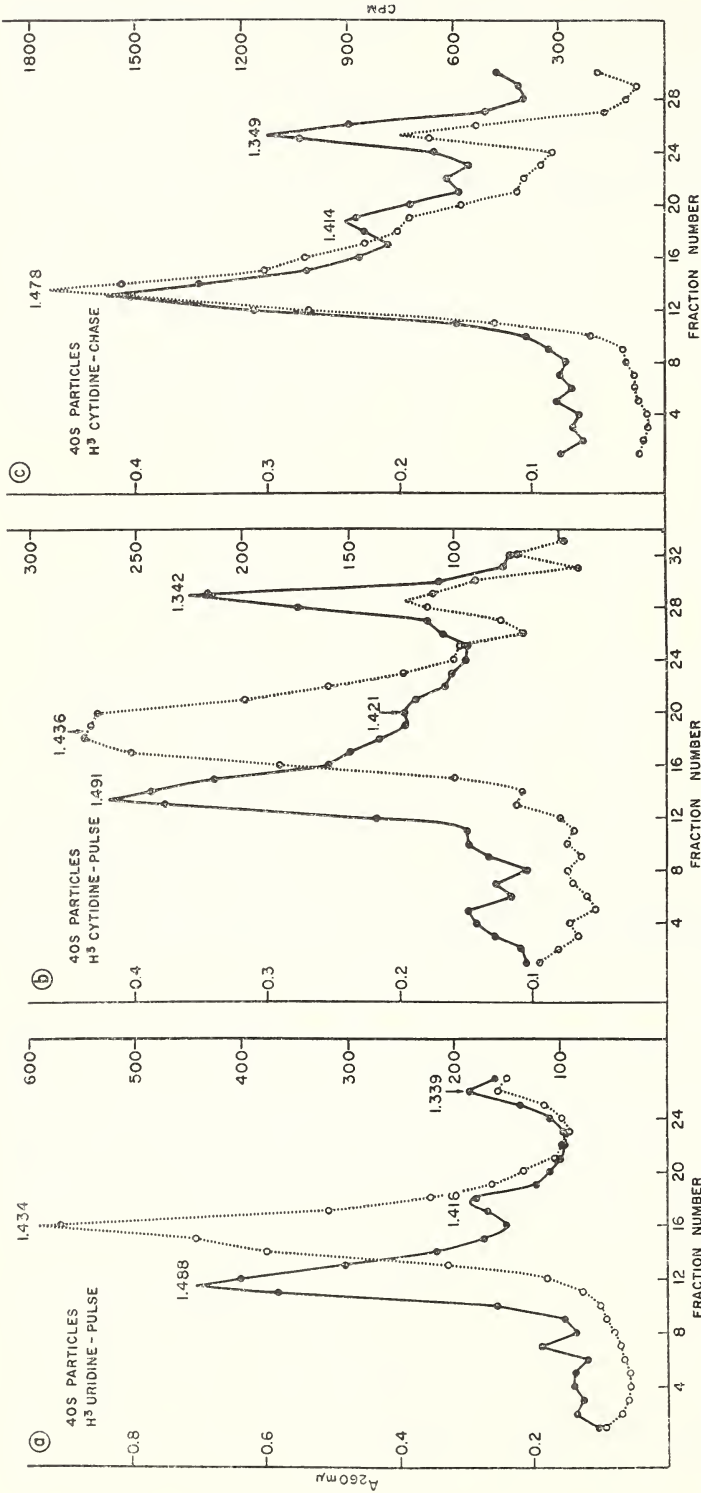
not rerun on sucrose before banding on CsCl so that it was contaminated by monoribosomes and 40S particles. These are represented by a shoulder on the main peak and the peak at $\rho = 1.49$. Rather unexpectedly, we observed an additional absorbance peak at approximately 1.39 g/cm^3 . With preparations of 40S particles (text-fig. 7b), the major density peak was consistently observed at about $\rho = 1.49$. Here again two minor peaks were found, one at roughly $\rho = 1.42$ and another at $\rho = 1.34$.



TEXT-FIGURE 7.—Preparations of RNP particles, isolated on sucrose gradients, fixed, and banded in preformed CsCl gradients for 10 hours at 35,000 rpm. $\rho = 1.31$ – 1.62 g/cm^3 . (a) 60S particles. (b) 40S particles.

Assuming that the major density peaks of the 40S and 60S particles represent subunits of the ribosome, and that the mass of the larger unit is twice that of the smaller, then the buoyant density values may be used to calculate the density of a monoribosome composed of one heavy unit and one light unit. The calculated value comes out to be 1.539 which is close enough to the measured value of 1.546 to encourage us that these densities are meaningful.

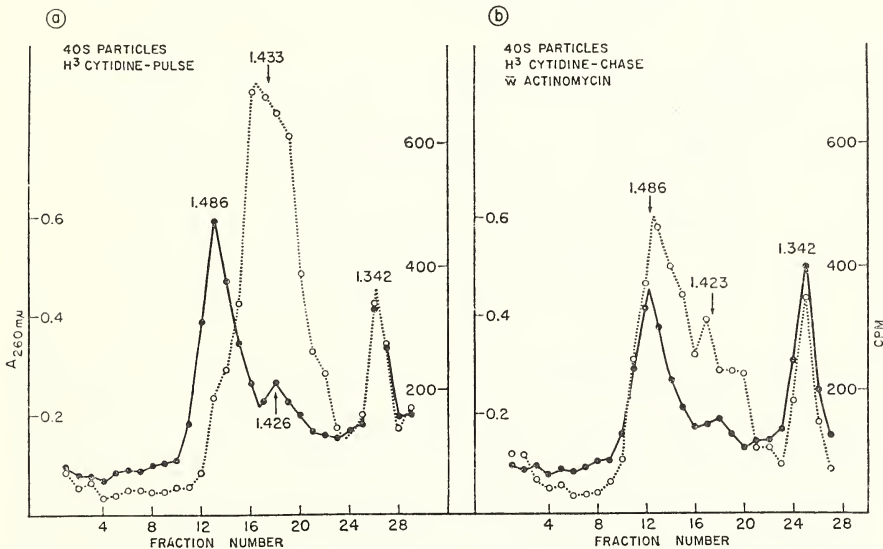
Now, what is the buoyant density of the rapidly labeled material which sediments in the 40S region? As can be seen in text-figures 8a and b, most of the radioactivity bands at a lower density than that characteristic of the 40S particles. Results with tritiated uridine (text-fig. 8a) and tritiated cytidine (text-fig. 8b) were essentially identical: In both cases there was a very prominent peak of radioactivity at about $\rho = 1.43$ and a smaller peak coinciding with the 1.34 absorbance peak. The relative



TEXT-FIGURE 8.—Sedimentation in CsCl of preparations of 40S particles from cells labeled with radioactive nucleosides. Isolation and density gradients as in text-figure 7. (a) Thirty-minute pulse with H³-uridine; (b) 30-minute pulse with H³-cytidine; (c) 6-hour chase in excess unlabeled cytidine [from a portion of the same population of labeled cells shown in (b)]. *Solid curves* (●): absorbance at 260 m μ . *Dotted curves* (○): radioactivity. The striking separation of radioactive and absorbance peaks seen with the pulse-labeled material is absent in the chased material. The total amount of radioactivity increases during the chase because of continued synthesis of RNA from nondiffusible pools of labeled precursor.

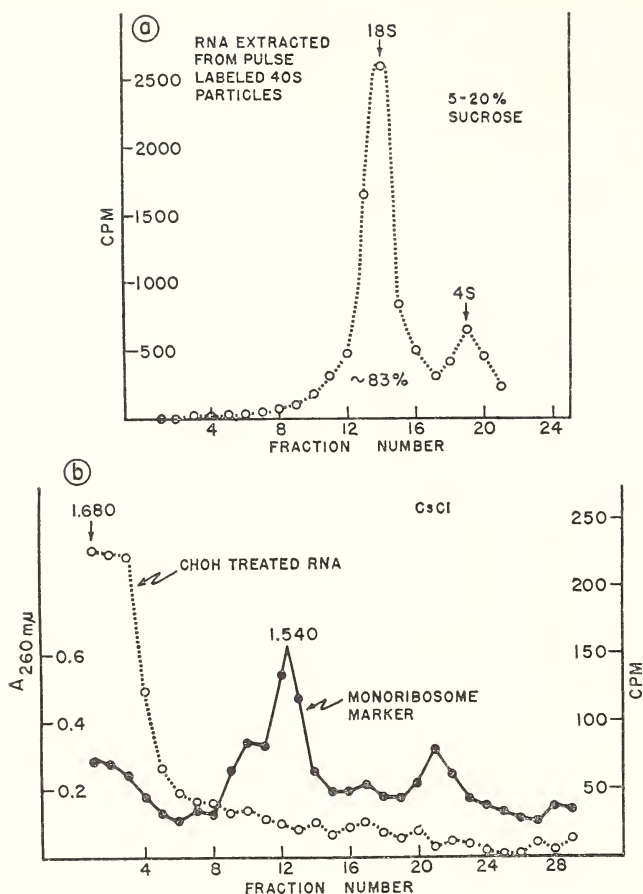
amount of radioactivity in the two peaks was somewhat variable, although with pulses of 30 minutes the $\rho = 1.43$ peak always contained the greater proportion.

In contrast to the above, with 40S particles from cells chased for 4 hours in the presence of unlabeled nucleoside, the radioactivity and the absorbance peaks coincide (text-fig. 8c). Thus the lower density property of the rapidly labeled 40S particle obviously represents a transient state: It disappears upon chasing. This is shown still more dramatically in text-figure 9, which represents the buoyant density analysis of the 40S particles from the experiment illustrated in text-figure 3. In this case, where the chase was carried out in the presence of actinomycin, the content of radioactivity in the 40S structures is practically the same in the pulse and chase. Here we observe a net loss of about 400 counts/minute in the $\rho = 1.43$ region which is compensated for by a gain of an equivalent amount of radioactivity in the $\rho = 1.49$ region. Interestingly, the amount of radioactivity in the 1.34 component differs little between pulse and chase.



TEXT-FIGURE 9.—Sedimentation in CsCl of 40S particles from cells labeled with H³-cytidine. Isolation and density gradients as in text-figure 7. (a) Thirty-minute pulse; (b) a portion of the same population of labeled cells chased for 2 hours with unlabeled cytidine and uridine in the presence of 0.4 μg/ml actinomycin D. *Solid curves* (●): absorbance at 260 mμ. *Dotted curves* (○): H³-radioactivity. *Note* that the prominent radioactive peak at $\rho = 1.43$ decreases almost threefold concomitant with the appearance of radioactivity in the $\rho = 1.49$ band.

In confirmation of earlier experiments (1), the majority of the rapidly labeled RNA extracted from 40S particles bands at 18S (text-fig. 10a). There is, however, some contribution—about 15–17%—which sediments in the lighter region. When the extracted RNA is banded on CsCl



TEXT-FIGURE 10.—RNA was extracted with 1% sodium dodecyl sulfate (SDS)-phenol from a preparation of *unfixed* 40S particles isolated from cells pulse-labeled for 30 minutes with H^3 -uridine. (a) Zonal centrifugation of RNA on a 5–20% sucrose gradient, 3 hours at 39,000 rpm. About 83% of the radioactivity sediments at 18S. (b) Same preparation of RNA shown in (a) was treated for 24 hours with 6% formaldehyde, mixed with a monoribosome preparation which provided a density marker, and centrifuged for 10 hours at 35,000 rpm in a preformed CsCl gradient, $\rho = 1.38\text{--}1.68\text{ g/cm}^3$. All of the RNA was concentrated in the bottom of the tube.

gradients together with a marker of monoribosomes, the RNA concentrates at the bottom of the tube (text-fig. 10b), proving that RNA extracted from formaldehyde-treated particles still possesses the heavy buoyant density characteristic of RNA. Thus we see that in the density gradients of text-figures 8 and 9 the radioactive peaks at $\rho = 1.43$ and $\rho = 1.34$ must represent true ribonucleoprotein structures, and not some free RNA extracted from the particles during the fixation process which exhibits a lower buoyant density by virtue of the formaldehyde treatment.

I want to make one final point concerning the existence of messenger RNA in the 40S region. Although the results of the buoyant density

Table 1.—Hybridization of RNA in cytoplasmic fractions

RNA source	Input of RNA		% of input cpm in hybrid
	μg	cpm	
40S particles (30-min pulse)	38	6100	2.0
	19	3050	2.6
	9.5	1525	3.3
CsCl banded 40S particles (30-min pulse):			
$\rho = 1.46\text{--}1.52$ g/ml	18	3300	4.5
$\rho = 1.39\text{--}1.45$	13	5900	4.7
$\rho = 1.33\text{--}1.38$	9	3400	19.8
40S particles (4-hr chase)	42	5390	2.5
Material sedimenting $\sim 25\text{S}$	15	6240	14.3

DNA agar containing 225 μg of DNA was incubated with RNA for 36 hours at 60 C in $2 \times \text{SSC}$. Hybridized RNA released at 70–75 C in $0.01 \times \text{SSC}$. RNA was extracted from the bulk preparations of 40S and 25S material with 1% SDS-phenol, and from the preparations which were fixed and banded in CsCl by incubation in 0.50 mg/ml pronase for 1 hour at 37 C followed by SDS-phenol.

studies did not support the idea of mRNA-subunit complexes, nevertheless we have been able to confirm the result of McConkey and Hopkins (12) that pulse-labeled RNA extracted from the 40S region hybridizes with DNA in measurably higher proportion than ribosomal RNA. The question is, then, with what kind of structure is this RNA associated? In an attempt to answer this question, we compared hybridization measurements on the RNA extracted from the bulk 40S particle preparation with RNA extracted from the various density components resolved on CsCl gradients, to determine whether a particular component is enriched with message. The results are shown in table 1. Using three different inputs of RNA extracted from 30-minute-pulse-labeled 40S particles, we obtained a percentage hybridization which is several times higher than we routinely find with rRNA. Whereas rRNA saturates under these conditions at less than 0.2% or 0.5 μg , here we were obviously not saturating the DNA with inputs as high as 38 μg or 17%. This clearly means that the preparations of 40S particles contain some RNA of message-like properties. We then measured the hybridization of RNA from 3 density fractions: the high density side of the major radioactive peak, $\rho = 1.46\text{--}1.52$; the low density side, $\rho = 1.39\text{--}1.45$; and the band at $\rho = 1.33\text{--}1.38$. The percent hybridization for both sides of the major peak was only slightly higher than for the bulk 40S preparations⁴ and was quite low compared to that found for the $\rho = 1.33\text{--}1.38$ band. The latter reproducibly ex-

⁴ Presuming that the RNA of the major density band represents pure 18S rRNA, one would expect this percent hybridization to be *less* than that found with the RNA from bulk 40S preparations. That this was not so may mean that the RNA was altered during the fixation and CsCl banding procedure so that it tends to form more unspecific hybrids with DNA. Nevertheless, the relative values for the various density peaks were quite consistent, indicating that comparisons from peak to peak are probably valid even though comparisons between fixed and unfixed material may not be.

hibited a very high percent hybridization even with inputs of 9 μg . This result indicates that the predominant concentration of messenger RNA in the 40S region is in the material of the $\rho = 1.33\text{--}1.38$ band. The percentage hybridization of the 40S particle-RNA from cells chased in unlabeled precursor for 4 hours was also higher than one would expect if these preparations contained only rRNA. Therefore it appears that even after a 4-hour chase there is still some labeled mRNA present in the 40S region. This correlates with the persistence of radioactivity in the $\rho = 1.34\text{--}1.38$ density band seen in text-figures 8c and 9b. It is noteworthy that RNA from structures sedimenting lighter than 40S ($\sim 25\text{S}$) also exhibits rather high values of hybridization. When the ribonucleoprotein from the 25S region was banded on CsCl, a single peak was observed at approximately $\rho = 1.37$, suggesting that this material may be similar to the mRNA-containing structures found in the 40S region.

We are still somewhat puzzled regarding the method of transport of mRNA from nucleus to cytoplasm. It appears that no significant portion of the rapidly labeled RNA from the 40S region is in the form of mRNA-subunit complexes, since no material with the predicted greater buoyant density is observed. One could argue that in our experiments we have stripped the 40S particles of mRNA which then becomes complexed with a large amount of protein to produce a particle of relatively low density. However, a simpler assumption might be perhaps that the low density particles are other structures which happen to sediment in the 40S region. They might be native structures analogous to "informosomes" (20-22) or they might represent nonspecific complexes of mRNA and protein.

On the other hand, our studies have led us unexpectedly to the discovery of some additional subribosomal components. One of these, the $\rho = 1.43$ component, seems to be a precursor to the native 40S particle. The nature of the structural alteration causing these newly formed particles to have buoyant densities lower than the mature particles is presently unknown. Since both types of particles have identical sedimentation behavior and both contain 18S RNA, it seems unlikely they contain different amounts of RNA or protein. The possibility that the distinctive buoyant densities are related to differences in solvent-binding capacity is currently being investigated. We are also endeavoring to learn whether an analogous situation holds for the newly formed 60S particles and whether we can detect any additional alterations in particle structure specifically associated with the transition from nucleolus to cytoplasm.

Note added in proof: Recent studies have shown that the newly formed 40S particles are specifically associated with additional protein components which may be removed by an appropriate incubation with trypsin (Perry, R. P., and Kelley, D. E., *Biochem Biophys Res Commun* 24: 459, 1966).

RESUMEN

Una de las principales funciones metabólicas asociadas con el nucleolo es la síntesis del ARN ribosómico y la fabricación de partículas ribosómicas.

La mayor parte de los datos disponibles están de acuerdo con el siguiente modelo. Los cistrones que codifican para el ARN ribosómico son transcritos en grandes moléculas precursoras 45S que son posteriormente degradadas dentro del nucleolo en los componentes 18S y 35S. Entonces, mediante un mecanismo todavía obscuro, los ARN se reúnen con la proteína en las subunidades ribosómicas. Las unidades recién formadas aparecen en el citoplasma como partículas 40S y 60S, que llevan ARN 18S y 28S respectivamente. Las partículas 40S son transportadas rápidamente al citoplasma, mientras que las unidades más grandes permanecen por un tiempo mayor en el núcleo, probablemente en el nucleolo. Sorprendentemente, las partículas 40S recién sintetizadas que aparecen en el citoplasma, se distinguen rápidamente de la mayor parte de las estructuras 40S nativas sobre la base de su distinta densidad de flotación. Se discute la significación del mecanismo de la formación ribosómica con referencia a su posible papel en el transporte de la información del núcleo al citoplasma.

REFERENCES

- (1) PERRY, R. P.: The nucleolus and the synthesis of ribosomes. Nat Cancer Inst Monogr 18: 325-340, 1965.
- (2) PERRY, R. P., SRINIVASAN, P. R., and KELLEY, D. E.: Hybridization of rapidly labeled nuclear ribonucleic acids. Science 145: 504-507, 1964.
- (3) BIRNSTIEL, M. L., SIRLIN, J. L., and JACOB, J.: The nucleolus: a site of transfer ribonucleic acid synthesis. Biochem J 94: 10-11, 1965.
- (4) SIRLIN, J. L., JACOB, J., and BIRNSTIEL, M. L.: Synthesis of transfer RNA in the nucleolus of *Smittia*. Nat Cancer Inst Monogr 23: 255-270, 1966.
- (5) WOODS, P. S., and ZUBAY, G.: Biochemical and autoradiographic studies of different RNA's. Evidence that transfer RNA is chromosomal in origin. Proc Nat Acad Sci USA 54: 1705-1712, 1965.
- (6) WOODS, P. S.: Autoradiographic studies of transfer RNA metabolism in *Vicia faba* root-tip cells. Nat Cancer Inst Monogr 23: 271-284, 1966.
- (7) RITOSSA, F. M., ATWOOD, K. C., LINDSLEY, D. L., and SPIEGELMAN, S.: On the chromosomal distribution of DNA complementary to ribosomal and soluble RNA. Nat Cancer Inst Monogr 23: 449-472, 1966.
- (8) PERRY, R. P.: Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. Nat Cancer Inst Monogr 14: 73-89, 1964.
- (9) GIRARD, M., PENMAN, S., and DARNELL, J. E.: The effect of actinomycin on ribosome formation in HeLa cells. Proc Nat Acad Sci USA 51: 205-211, 1964.
- (10) GIRARD, M., LATHAM, H., PENMAN, S., and DARNELL, J. E.: Entrance of newly formed messenger RNA and ribosomes into HeLa cell cytoplasm. J Molec Biol 11: 187-201, 1965.
- (11) BIRNSTIEL, M. L., CHIPCHASE, M. I. H., and HYDE, B. B.: The nucleolus, a source of ribosomes. Biochem Biophys Acta 76: 454-462, 1963.
- (12) MCCONKEY, E. H., and HOPKINS, J. W.: Subribosomal particles and the transport of messenger RNA in HeLa cells. J Molec Biol 14: 257-270, 1965.
- (13) JOKLIK, W. K., and BECKER, Y.: Studies on the genesis of polyribosomes. I. Origin and significance of the subribosomal particles. J Molec Biol 13: 496-520, 1965.
- (14) PERRY, R. P.: Unpublished observations.
- (15) RAKE, A. V., and GRAHAM, A. F.: Kinetics of incorporation of uridine-C¹⁴ into L cell RNA. Biophys J 4: 267-284, 1964.
- (16) HADJIOLOV, A. A.: Biochim Biophys Acta 119: 547-556, 1966.
- (17) PERRY, R. P., and KELLEY, D. E.: Buoyant densities of cytoplasmic ribonucleoprotein particles of mammalian cells: distinctive character of the ribosome subunits and the rapidly labeled components. J Molec Biol 1: 255-268, 1966.
- (18) SPIRIN, A. S., BELITSINA, N. V., and LERMAN, M. I.: Use of formaldehyde

- fixation for studies of ribonucleoprotein particles by cesium chloride density gradient centrifugation. *J Molec Biol* 14: 611-615, 1965.
- (19) HENSHAW, E., REVEL, M., and HIATT, H. H.: A cytoplasmic particle bearing messenger ribonucleic acid in rat liver. *J Molec Biol* 14: 241-256, 1965.
- (20) SPIRIN, A. S., BELITSINA, N. V., and AITKHOZIN, M. A.: Informational RNA of early embryos. *Zh Obstch Biol* 25: 321-338, 1964.
- (21) NEMER, M., and INFANTE, A.: Informosomes: messenger RNA-protein complexes. *Fed Proc* 24: 283, 1965.
- (22) SPIRIN, A. S., and NEMER, M.: Messenger RNA in early sea urchin embryos: cytoplasmic particles. *Science* 150: 214-217, 1965.

DISCUSSION

Brown: In your table 1, why does the RNA from the CsCl-banded, formaldehyde-treated particles hybridize to a greater extent than RNA from the original 40S particles? Were these equivalent inputs?

Perry: I intended them to be equivalent inputs and they were almost the same. I don't know whether the difference between the 4.5-4.7% for the $\rho=1.39-1.52$ components and the 2.6-3.3% for the whole 40S particle is real. We intend to repeat these measurements because we also find this difference puzzling.

Brown: In your pulse chase experiment with actinomycin (text-fig. 3), only the 40S peak was labeled after the pulse; yet after the chase the monosome and the 60S peaks have the same specific activities, and the 40S peak is still very high. This must mean that, under these conditions, the 40S particle was not incorporated into the monosomes; that is to say that the only radioactivity in the monosome peak after this 2-hour chase was contributed by 60S particles.

Perry: No, I don't think you can conclude this. Remember that we don't know the amount of 40S particles still in the nucleus at the end of the pulse. We are looking only at the cytoplasmic particles and, therefore, there could still be labeled 40S particles coming out of the nucleus during the chase. This would prevent the 40S specific activity from dropping down to a value comparable to that of the other components.

Brown: Then the equal specific activities of the monosome and 60S subunit would really be a coincidence and have no real meaning?

Perry: I think so. If you extend the chase, the specific activity of the 40S peak eventually falls to the level of the other particles. The actinomycin doesn't block the migration; it only blocks the new formation of 18S RNA.

Feinendegen: Does the total activity of all particle fractions remain stable after chasing with cold nucleoside? I ask this because we know the pool is not easily diluted with chaser.

Perry: When chasing without actinomycin, one gets a large increase in incorporation into the various components. Of the two sets of cesium chloride gradients that I showed, the first one (text-fig. 8) was without actinomycin, and in this case the radioactivity in the 40S particle went up by a factor of 6 between the pulse and the chase. As you say, this is because there is a large nucleotide precursor pool which cannot be diluted out by the addition of cold nucleoside. But in the second set (text-fig. 9), we added actinomycin in addition to the unlabeled nucleosides. In this case the total counts in the pulse and chase were essentially the same.

Vincent: You spoke of migration of 40S and 60S particles from the nucleus. What evidence do you have that 40S and 60S particles migrate from the nucleus? I have seen no evidence in what you have just presented or in your previous papers that the RNA found in the cytoplasm in these particles emerges in a 40S or 60S particle rather than in a smaller unit which may be immediately coated with protein.

Perry: Perhaps we should delay the question until Dr. Penman has had a chance

to present material which may be pertinent to this point. If you wish, we can take up this problem again as a part of the Panel Discussion.

Taylor: Would you briefly describe the treatment with formaldehyde and then how the RNA is later extracted from the particles?

Perry: We add the formaldehyde, buffered to a pH of 7.8, at 3 C to the fractions of particles taken right off the sucrose gradient. The final concentration of formaldehyde is 6%. We allow these to fix for 24 hours at 3 C and then dialyze out the formaldehyde and the sucrose and put the fractions into preformed cesium chloride gradients. After appropriate centrifugation we take aliquots for monitoring the band positions, and then after removal of the CsCl by dialysis we extract the RNA from the fixed particles with pronase, sodium dodecyl sulfate, and phenol. The pronase digestion is for 1 hour at 37 C with 500 μ g per ml of enzyme.

Taylor: Does the RNA and protein of a particle separate completely in cesium chloride if you do not pretreat with formaldehyde or some cross-linking agent?

Perry: Yes. One interesting fact I should mention is that the ability of the high salt to separate the nucleic acid from the protein is greater in the case of the small particle. The 40S is the most sensitive, the 60S next, and the monoribosome is the least sensitive. If one studies the degradation of unfixed particles as a function of time in cesium chloride, one observes a very rapid separation of RNA and protein of the 40S particles and a much slower process in the case of the monoribosome. The latter are further stabilized in the presence of a relatively high concentration (0.04 M) of Mg^{++} .

Panel on Ribosome Biogenesis¹

Chairman: HERMAN LEWIS

Panel: M. BIRNSTIEL, D. BROWN, J. GALL, S. PENMAN, R. PERRY (*Moderator*), and W. VINCENT

Lewis: The first portion of the session will consist of discussion of various topics related to the biogenesis of ribosomes. The moderator for this part of the session will be Dr. Perry. After each member has had a chance to make comments and throw questions at each other, the entire audience will be permitted to direct questions to the panel on the subject of ribosome biogenesis. We will have a 15–20-minute discussion on this topic and then the remaining time in this session will be open discussion in which the audience can address their questions to any member of the panel or to anyone in the audience on matters related to anything discussed in the Symposium thus far. Without further ado I will turn the session over to Dr. Perry.

Perry: Thank you. To get this panel under way I will start by considering the problem of the transformation of the ribosomal RNA (rRNA) precursor, the 45S nucleolar RNA, into the 28S and 18S rRNA components that exist in the cytoplasm. This particular point was one that Dr. Busch, who unfortunately could not join us for the panel, stressed rather strongly in his presentation. I will summarize his views and then ask one of the panel members to comment on it. In his talk Dr. Busch said he does not believe nucleolar 45S RNA is a precursor to the 18S rRNA. I discussed this matter with him a little further. He said he believes there are basically three different kinds of 45S RNA molecules: first, the 45S RNA molecule which is a member of the heterogeneous DNA-like RNA that Dr. Penman showed and that I commented on; second, also one I think most of us accept, the precursor of the 35S RNA which finally becomes the 28S rRNA component; third, a 45S RNA, present in Dr. Busch's nuclear ribonucleoprotein (RNP) network, which he thinks is the precursor of 18S rRNA. Dr. Busch said he believes this third 45S RNA is really present on the periphery of the nucleolus, and that in his particular method of preparation of nucleoli it is dislodged

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

and appears in the nucleoplasm. Now this would be a way of resolving something that seemed completely contradictory to the notion that the 18S rRNA is of nucleolar origin. However, it does assume as a basic premise that there are two distinct 45S RNAs, polycistronic if you wish—one, a precursor of 28S rRNA and another, a precursor of the 18S rRNA component. I would like to ask Dr. Birnstiel, who I know has thought a good deal about this, if he would like to comment on the probability of there being two distinct 45S molecules, one for 18S RNA and one for 35–28S RNA, rather than a 45S RNA containing both the 18S and 35–28S RNA components.

Birnstiel: Dr. Busch's belief is based on the fact that 45S, 35S, and 28S RNA share the same GC content, but 18S RNA does not. In our hybridization experiments we have, as yet, found no suggestions that the 28S and 18S rRNA cistrons are contiguous, although we intend to examine this point more thoroughly. The 45S RNA as a composite of 28S and 18S rRNA has provided an excellent working hypothesis and I feel that the burden of disproving its validity lies with the nonbelievers.

Penman: I think Dr. Birnstiel has stated the problem very well. I gather that Dr. Busch's premise is based on the fact that he also doesn't see any 16S or 18S RNA in the nucleolus, although it is present in his nuclear residue fraction which contains the nuclear RNP network. I do not think this is a sufficient reason to assume there is a different site for the formation of 18S RNA.

Vincent: I want to insert a cautionary note. When one examines a complex system such as that demonstrated on sucrose gradients of total cell RNA, and then makes kinetic analyses of what appears to be the flow of label through this highly structured system of many compartments, one may fall into unsuspected error. Not long ago we examined rapidly labeled RNA in yeast cells (Kitazume, Ycas, and Vincent, *Proc Nat Acad Sci USA* 48: 265–282, 1962) and could show that the kinetics of labeling proved this fraction must be the precursor of rRNA. I think there is no question with respect to the calculations or the data; it just happens that the cell does not work that way. I would like to suggest that interpretations of apparent flow of label in other systems might be examined for the possibility of similar sources of error.

Penman: I would like to comment on that. There are experiments I did not describe since I don't have the data with me, but I think they are good enough to be pertinent here. The reason we do kinetic measurements, of course, is that we can't chase nucleotides rapidly. Since the nucleotides don't exchange with the outside pool, we are forced to watch the progressive migration of label. However, as I mentioned, all of the methylation in high molecular weight RNA is initially in the 45S nucleolar RNA. One can chase methionine. When one watches the chase of methylated RNA through these various species, the methylated RNA does exactly what is expected—it is chased out of the 45S RNA region of gradients. It is only the 45S RNA that is methylated in short pulses.

Incidentally, this means the transition of 35S RNA to 28S RNA is not due to methylation.

Brown: The two aforementioned theories predict two different gene arrangements: one is blocks of 28S rRNA cistrons and then blocks of 18S rRNA cistrons; the other is alternating 28S and 18S rRNA cistrons. If they are alternating, one would expect the degree of hybridization would be relative to the molecular weights of the two species, that is to say, if 28S RNA is 3 times the molecular weight of 18S RNA and they are alternating, then the percent of hybridization should be 3 to 1. But as Dr. Birnstiel pointed out, in *Xenopus* they are not 3 to 1; in fact, 28S rRNA hybridizes in *Xenopus* to 0.07% of the genome and 18S rRNA to 0.04% of genome.

Penman: Do I understand you correctly? Do you think that 0.07 to 0.04 is significantly different from 2 to 1? I thought the difference in molecular weight was about 2 to 1.

Brown: My understanding is that the molecular weights of 28S and 18S RNA are 1.6 and 0.6×10^6 , respectively.

Penman: I've heard that too, but the ratio of optical density or the ratio of radioactivity, which is about the same thing, is certainly nowhere close to 3 to 1; it is close to 2 to 1. I've never understood where the data on molecular weight come from [Insert by editors: Petermann, *The Physical and Chemical Properties of Ribosomes*, Elsevier, 258 pages.]

Perry: I think the number usually given is about 2.3 to 1 for the absorbance, and so I would tend to agree that the ratio of hybridization for the two cistrons, the 28S to the 18S rRNA, is not quite as high as one would expect. But I feel the discrepancy is not sufficiently great to draw any strong conclusions.

Birnstiel: I agree. If you look through the papers by people working on hybridization, they will always caution you that these values are just approximations, and I really think it should not be used as an argument against the theory of alternating ribosomal cistrons.

Perry: The second point on our agenda concerns the role of methylation in the conversion of 45S RNA to its final products. From what Dr. Penman has said, it appears that methylation is not involved here because the methylation probably occurs at the 45S level. Would anyone on the panel like to comment on that? If not, we can go on. What is the cellular site of the 35S to 28S RNA transition? It seems to me that in your presentation, Dr. Penman, you have fairly good evidence that the 35S to 28S RNA transition occurs in the nucleoplasm. Is this correct?

Penman: Well, no. The data indicate that you see 35S RNA in the nucleolus and 28S RNA in the nucleoplasm. Exactly where the transition takes place one cannot say.

Gall: On that point I would just like to remind you that the main optical density and radioactivity peaks which I find for RNA from oocyte nuclei are at 30S. This material definitely accumulates in the nucleus.

Perry: But that still wouldn't tell you where the site of transition is.

Vincent: Does anyone have any idea as to what the nature of the transition from 35S to 28S RNA might be? Is it some sort of configurational change, giving an apparent shift in sedimentation velocity, or is it due to an actual change in molecular size by the loss of polynucleotide fragments?

Brown: There is a very good explanation for this transition. There is a ribosome-associated piece of low molecular weight RNA, which has been described by Dr. Comb and Dr. Woods. It is estimated to be about 100 nucleotides in length. We and others have always found this RNA associated with ribosomes. Furthermore, it is synthesized coordinately with rRNA. This class of RNA is not synthesized by the anucleolate mutant of *Xenopus*, suggesting that it is actually part of the rRNA operon. In addition, Dr. Comb says that it is associated with the 60S ribosome particle. Therefore, a 35S RNA molecule might cleave to form one 28S and one small piece of RNA, both of which become included in the 60S ribosome subunit.

Birnstiel: I would like to go back to the discussion of the nucleoplasmic site of transformation of 35S into 28S RNA, if I may. If one looks at the labeling patterns of cell components by autoradiography, it is clear that the nucleolus is the major source of cytoplasmic rRNA. There has been no indication, so far, that a nucleoplasmic pool mediates the relationship between the nucleolus and the cytoplasm. In view of what has been said at this Conference it might well be worthwhile to re-examine this point.

I have a suspicion that a nucleoplasmic pool of RNP particles containing 28S RNA might be artifactual and derive from elution and mechanical abrasion of truly nucleolar constituents during the fragmentation of the nuclei. This is especially suggested from our experiments on isolated pea nucleoli in which we were able to show that a loss of RNA from isolated nucleoli always entailed a loss of the particulate rim of the nucleoli and the concomitant accumulation of RNA or RNP in the nucleoplasm (Birnstiel, Rho, and Chipchase, *Biochim Biophys Acta* 55: 734-740, 1962; Birnstiel, Chipchase, and Hyde, *Biochim Biophys Acta* 76: 454-462, 1963). I wonder to what extent the so-called nucleoplasmic RNP particles in other systems represent constituents released from nucleoli during isolation.

Penman: Dr. Birnstiel's comments are absolutely right to the point. However, I would like to ask a question about technique. If you look at the amount of 28S RNA which is presumably in a 60S RNP particle in the nucleoplasm, you can see that the chromatin-associated RNA has much more label in it by the time this particle becomes radioactive. The 28S RNA in the nucleoplasm does not begin to label for 45 minutes. By this time, there is at least 10 times as much radioactivity in chromatin-associated or polydisperse nuclear RNA. Could autoradiography pos-

sibly distinguish whether 10 or 20% of the radioactivity in the extra-nucleolar nuclear fraction is in the RNP particles?

Perry: I hesitate to cut off this discussion but I would like to touch on a few more points. Something of interest to all of us is the origin of ribosomal protein: What are its sites of synthesis and what are the sites of ribosome assembly? One could conceive that these two might be different. The synthesis of the ribosomal protein could occur in the cytoplasm, be transported to the nucleus, and there be assembled with the RNA to form ribosomal particles; or, on the other hand, it could be synthesized together with the RNA at the nucleolar site. One theory supported by some recent work with bacteria (Nakada, *J Molec Biol* 12: 695-725, 1965) is that the rRNA is itself the template for the synthesis of the ribosomal protein. At this point one should consider whether the rRNA contains sufficient information to code for the ribosomal protein. There appears to be a paradox. From the number of bands formed on acrylimide gels after lithium chloride treatment or some equivalent procedure, it is estimated that there are from 25 to 35 different types of ribosomal protein. Therefore, it seems unlikely there is very much redundancy in the amino acid sequences, leading one to predict that there are unique codon sequences for the bulk of the ribosomal proteins. Using a coding ratio of 3 bases to 1 amino acid and the relative average molecular weights of nucleotides and amino acids, one can calculate how much RNA would be needed to code for these proteins if they were all different. The value obtained is at least 3 times greater than that present in the total rRNA. This is disturbing if one wants to insist that rRNA specifies all the ribosomal protein. To reiterate the question: What are the sites of synthesis of ribosomal protein and is there a chance that the rRNA could be a template for the synthesis of its own protein?

Birnstiel: It is true that the rRNA can only code for one third of the proteins present in the ribosome. But in the system with the fewest ribosomal cistrons known so far, namely, *Bacillus subtilis* with three (Oishi and Sueoka, *Proc Nat Acad Sci USA* 54: 483-491, 1965), there is enough over-all information to produce ribosomal proteins, provided that there was an exchange of the different protein species produced on the rRNA templates.

Brown: Are you assuming that each rRNA cistron is unique?

Birnstiel: Each rRNA cistron could be different and the proteins they produce could be unique. In higher organisms the 45S RNA might represent such a message, and it is interesting to see that in Suskind's experiments the suppression of the appearance of the 45S ribosomal precursor RNA in the nucleolus by actinomycin also abolished a good proportion of the nucleolar amino acid incorporation (Suskind, *J Cell Biol* 24: 309-316, 1965). Dr. Perry, have you been able to confirm this in your system?

Perry: I tried this type of experiment several years ago. Unfortunately, with the cell cultures I have studied, I do not find situations such as Suskind does where the amino acid incorporation into nucleoli is signifi-

cantly higher than that of the rest of the cell, even though our cells are cultured under relatively similar conditions. I don't know if this is a difference in cell strain or something else. I don't have confidence in ascribing autoradiographic grains over nucleoli to nucleolar protein synthesis because I usually have such a high background of cytoplasmic incorporation at all times—after pulses, chases, and under all conditions of actinomycin treatment. Therefore, I cannot confirm Suskind's observation.

Vincent: In some experiments we have been doing on synchronous cultures of yeast, we find that most ribosomes are synthesized during a relatively short segment of the cell cycle. This finding led to some interesting experiments to test the conclusion which Dr. Brown has described, namely, that the nucleolus is present only when ribosomes are being synthesized. Another observation we have made in this system is that no matter how short a period of labeling with RNA precursor we use (*e.g.*, 1 minute in 180-minute doubling time), we find newly synthesized RNA in 80S ribosomes. This suggests that there may be a pool of preformed ribosomal proteins. In preliminary experiments with this system where we can turn on ribosome synthesis after long periods of no synthesis, we have found that newly synthesized rRNA is formed into ribosomes in the absence of any apparent new ribosomal protein. This suggests that these cells contain a conserved pool of ribosomal protein, and, in addition, raises the problem of just where ribosomal proteins may be made.

Perry: There is some very good corroborative evidence on a pool of protein from Dr. John Warner's work (personal communication). Since Dr. Penman was formerly in the same laboratory, I would like for him to comment on this and also, if possible, on whether there are different types of ribosomal proteins with different kinetic parameters.

Penman: Well, it is difficult to comment on Dr. Warner's work. I hope I can do him justice and I'll be very limited in my comments. It is my understanding that Dr. Warner has been doing some very precise experiments on the proteins associated with the 50S ribosomal subunit. He has found that one must be very careful in isolating the ribosomal subunits to clean them of aggregated proteins, a point that has come up here before. He doesn't find too many bands, perhaps 15 or 16. This finding has bearing on the previous comments about how much message is necessary for the ribosomal proteins. He finds that the protein does not go with the RNA, I believe, but it appears as though there is a pool of preformed proteins present. We have also done an interesting experiment which has bearing on several of the subjects mentioned. Actinomycin blocks the movement of RNA to the cytoplasm, as we have seen. Now, why does it do this? Is it because there is a short-lived message which stops making protein so that the ribosomes can't be finished and come into the cytoplasm? Well, if one uses actidione which allows continued uridine incorporation but completely inhibits protein synthesis, one finds that, in fact,

a lot of RNA moves to the cytoplasm, so that the actinomycin is not simply blocking a short-lived messenger RNA which makes protein. I wonder if this small piece of RNA that Dr. Comb and Dr. Brown have talked about might be involved in this process. Perhaps a piece of small RNA is necessary for ribosomal completion, and if it doesn't go with the same ribosome, *i.e.*, if the ribosomes exchange these as they are forming, then the actinomycin may be blocking the formation of a piece of RNA necessary for ribosome completion and maybe that's why things hang up in the nucleus. One other comment on the question of actinomycin decay of nuclear protein synthesis. We have looked at that and there seems to be a rapid but not extensive decay of nuclear protein synthesis with actinomycin. This phenomenon could conceivably be linked to the rapid decay of the polydisperse chromatin-associated RNA.

Perry: In your talk, Dr. Penman, you made the statement that nuclei are active in protein synthesis and then you said they don't have the small ribosomal subunit. Thus you gave us an indication that here was, indeed, a new system which could function without the conventional ribosomes. Now you must say something on that.

Penman: I don't know what to say.

Perry: Do you think it might be something like the soluble system described for bacteria (Kaji, Kaji, and Novelli, *J Biol Chem* 240:1185-1191, 1965) ?

Penman: There is an extensive literature on mitochondrial protein synthesis. Mitochondria appear to synthesize what are apparently structural proteins and also to contain few, if any, ribosomes.

Gall: There are probably ribosomes in mitochondrial preparations.

Penman: Not very many. For instance, one can get mitochondria out—I'm getting awfully far afield—of HeLa cells which do not have extensive endoplasmic reticulum and no membrane-bound ribosomes in the cytoplasm. One can get these mitochondria fairly clean and it can be seen that there is very little 16S and 28S RNA, if any, in this fraction. We have looked at nuclear protein synthesis, but I can't possibly talk about this without presenting the data. There seems to be something new, but it is too startling to talk about without some really solid evidence, which I don't have in hand.

Perry: I believe Warner has found two kinetically distinct types of protein, one that is incorporated rapidly into the ribosome and another that goes on at a slower rate. I think this is rather important. Many of you probably are familiar with the ribosome models of Spirin [Abhandl Deut Akad Wiss Berlin, *Kl Med* 1964(4), 163] where he postulates the existence of two types of proteins, a very tightly bound structural protein associated with rRNA and another protein that is bound in the presence of Mg^{++} and is responsible for stabilizing the conformation of the particle. Would anyone like to comment on different types of ribosomal protein?

Penman: Well, I do know something about this aspect of John Warner's work. There are really three classes of ribosomal protein. There are junk proteins that are picked up if one is not careful about the ribosomal isolation. These become labeled very rapidly. If one uses a 5-minute amino acid pulse and then a chase, every ribosome immediately has some radioactivity which can be eluted if one washes the ribosomes in a high ionic strength buffer. There is also a relatively rapid incorporation of radioactive amino acids into ribosomes and then a relatively slow incorporation. What this has been taken to mean is that there are proteins made in the cytoplasm that truly associate with ribosomes. For instance, the transferase is possibly not a ribosomal constituent when it is synthesized. The slow emergence of labeled ribosomal protein from the nucleus into the cytoplasm is thought to be the true structural protein coming out with the RNP particles.

Perry: Thank you. I think we should go on to the next topic. This concerns the exit of RNP particles from the nucleolus. Now I know that Dr. Vincent is a very strong antagonist to the idea that there is any exit of RNP particles from the nucleolus, and yet, on the other hand, Dr. Penman has presented convincing evidence that essentially none of the RNA of the nucleus permanently remains there. That is, if one chases a pulse of labeled RNA precursor for a sufficiently long time, all of the radioactive ribosomal precursor RNA disappears from the nucleus. I really should allow Dr. Penman to comment on his own work, but I want to make the point that we are talking about the 28-30S RNA which has been shown in peas (Birnstiel, Chipchase, and Hyde, *Biochim Biophys Acta* 76: 454-462, 1963) and in animal cells (Girard, Penman, and Darnell, *Proc Nat Acad Sci USA* 51: 205-211, 1964) to be extractable from nuclei as part of a 60S particle. Therefore, on the basis of this evidence, it seems to me we can assume that the RNP particles exit from, rather than permanently remain with, the nucleolus. Of course, we should remember that a 4-day chase spans 4 generations and one could still argue that all the dispensing of these particles occurs at mitosis when the nucleolus disintegrates. Dr. Penman didn't mention this point, but probably many of you were thinking about such a possibility.

Penman: As I mentioned parenthetically, the high molecular weight RNA methylation occurring only on the ribosomal precursor RNA shows a washout time from the nucleolus on the order of 90 to 120 minutes. Thus, the nucleoli don't have to disappear for at least that RNA component, the methylated component, to disappear. Now whether there is longer lived material that is 30S and not methylated, of course, I don't know.

Vincent: I think Dr. Perry misquotes me with respect to what I think about rRNA-containing particles coming out of the nucleus. What I have been trying to get clear information on is whether intact ribosomal precursor subunits consisting of both RNA and protein have been demonstrated in the nucleus, and whether such particles have been demonstrated

to be the precursors of cytoplasmic ribosomes. With respect to the question raised by Dr. Perry regarding the dumping of new ribosomes or ribosomal RNP precursors into the cytoplasm at mitosis, I can point out that in our yeast system the maximum rate of new ribosome appearance takes place just before, or at the time of, prophase in the yeast cell.

Perry: Regarding the point about whether the rRNA comes out in the form of particles, if one observes what is coming out into the cytoplasm, one finds that the only rRNA emerging is in the form of particles. This seems relatively certain from a whole host of evidence and I don't believe anyone on this panel would really disagree with that point. One cannot demonstrate free rRNA coming out into the cytoplasm. One can demonstrate rRNA coming out and it is all in particles. Now that seems to me a rather strong and incontrovertible argument. Would you like to comment on that?

Vincent: I don't accept this argument as necessarily relating to ribosomes, because one doesn't detect a free high molecular weight RNA any place in the cytoplasm.

Birnstiel: I am slightly puzzled by the contortions of my colleagues to explain away all 18S RNA from the nucleus and nucleolus. I would like to look at the question of the intranuclear pools of 18S RNA in a slightly different way. If we assume that in an exponentially growing cell culture the rRNA is doubled under steady-state conditions throughout the interphase of each cell, one can make some interesting predictions. First, it becomes evident that under these (grossly simplified) conditions, the transition time of the different components of the reaction chains relates very simply to the pool sizes in the different cell compartments and vice versa. According to Dr. Penman, the pool of 45S RNA and HeLa cells corresponds to some 2% of all cellular rRNA, that of 35S and 28S RNA combined to some 5%. With an interphase period of about 20 hours, these pools calculate to an intranuclear transition time of 20 minutes for the 45S RNA and some 60 minutes for the 35S and 28S RNA combined, together some 80 minutes, which is in good agreement with the experimentally detected appearance of the 28S rRNA in the cytoplasm after 1-1½ hours.

Dr. Busch measured the half-life of 45S RNA in rat liver and independently determined it to be some 8-10 minutes which corresponds to an average mean survival time of the 45S RNA molecule of some 12-15 minutes, which compares favorably with the value derived from the 45S pool sizes. So far the calculations have worked out quite well. Let us now turn to the 18S RNA. It is generally assumed that the 18S RNA is derived at the time when the 45S is converted to 35S RNA. The 45S RNA possesses a transition time of some 20 minutes, and yet the 18S rRNA appears in the cytoplasm only after 30-40 minutes. There is then a discrepancy which is not compatible with our assumption that 18S arises directly, *i.e.*, without further intermediates, from the breakdown of 45S RNA and the absence of a measurable 18S RNA pool within the nucleus.

The above considerations provide, however, an easy rationalization of why the intranuclear 18S RNA pool might well be considerably smaller than that of the 35S and 28S RNA combined.

Penman: Dr. Birnstiel, I would like to point out that there is no apparent contradiction between the short half-life of 45S RNA and the labeling kinetics. That is to say, one is not looking at the same thing when he looks at optical density and when he looks at counts. Perhaps I can illustrate this in the following way. It looks like the procedure of going from 45S RNA to finished rRNA is a very ordered one; there is no suggestion of pools or exponential behavior, etc. Everything appears to occur in a linear manner. Apparently, 25 minutes is the absolute minimum time for the first labeled nucleotide to go from its incorporation into 45S RNA to the cytoplasmic 18S rRNA and the nucleolar 35S RNA, at least in HeLa cells. Now, suppose there is an assembly line with 45S RNA in various states of maturity, and when a 45S RNA molecule finally matures, then it's cleaved. Let's assume, just for discussion, that it is cleaved into a 35S RNA which stays in an organized state in the nucleolus and an 18S RNA which is exported. When one looks at label, then, one measures the maturation time and this takes 25 minutes after which 35S and 18S RNA appear simultaneously, 18S rRNA in the cytoplasm and 35S RNA in the nucleolus. If one looks at optical density, however, one is looking at all of the 45S molecules. When actinomycin is added, new synthesis of 45S molecules is blocked. But the existing 45S RNA molecules seem to continue their process of maturation and are continuously leaving the 45S peak. Thus, the 45S OD begins to decay immediately upon the addition of actinomycin, even though it takes 25 minutes for labeled RNA to reach the point where it leaves the 45S peak. As to the question of why one doesn't see 18S RNA being exported, first of all, perhaps this reflects the isolation procedures we use, although Dr. Gall and I use quite different techniques. It is possible, in fact, we just wash it out of the nucleus during preparation. However, this may not be the case and our experiments may reflect the *in situ* situation. One can't make a prediction about this kind of thing because one parameter missing is the exit time of 18S RNA from the nucleus. If the exit time is one millisecond, then one would see no counts in the 18S RNA region in the nucleus. If the exit time is very long, then one would expect to see counts. The exit time for 35S RNA is quite long because apparently it is being processed.

Perry: I want to make one final comment with regard to the question of exit time. Might the reason why the nucleolus looks like a very well-defined organelle be that there is a slow release and a fast transport time of RNP particles from it? After all, a very pertinent question to ask about all this is: If nucleoli are a source of ribosomes being transported to the cytoplasm, why aren't the particulate elements of the nucleolus arrayed like a Gaussian distribution function? Why doesn't one see wider and wider circles of more and more dilute particles? It doesn't

look like this; it has a very sharp boundary. Of course, there could be morphological reasons for this, but I don't want to go into that. One possible explanation, however, could simply involve different rates of release and transport of the particles. If once the particle is released it goes out very fast, say in the order of milliseconds, this would result in a very sharply delineated structure.

I have to close this part of the discussion so that we may entertain questions from the audience. We will start out with questions on ribosome biogenesis, but I think if time permits we should consider any question that has come up in the Conference which needs clarification. I now turn the session over to the Chairman. He has given us permission to continue for about 15 minutes.

* * *

Lewis: The floor is open. I ask you first to state your own name and then the name of person to whom you are directing the question.

Taylor: I would like to go back just for a moment to the short discussion on the release of ribosomes in relation to phases of the cell cycle. It seems to me that the panel left the question in a very unsatisfactory state. There must be some regular transport mechanism for moving ribosomes or rRNA from the nucleus for we certainly have several kinds of evidence that rRNA leaves the nucleus during interphase. This is especially evident in an oocyte where one can follow the movement autoradiographically.

Perry: If I understand your question, you want to compare the release in mitosis with the release during interphase. Well, the amount released in mitosis is trivial compared to the amount that has to be released during the cell cycle. The fraction of total RNA in the nucleolus versus that which has to be made in one generation time in order to supply the cell's complement of ribosomes is a trivial quantity.

Birnstiel: I do not think that the amount of RNA within the nucleolus and on the way to becoming rRNA is trivial. In an exponentially dividing cell culture it should amount to some 5-10% of the cellular rRNA.

Perry: Five to 10%? Well, I regard that as small—it's a subjective matter: what is large and what is small.

Sirlin: I address the question of very rapid transfer or exit from the nucleus generally to the panel. It is a "bread-and-butter" observation in polytene nuclei that if one lets the label go high and then inhibits in parallel experiments either the nucleolus with actinomycin or the chromosomes with benzimidazoles, one can observe patches of RNA activity in what we will call the nuclear sap (Sirlin *et al.*, this Symposium). There is no doubt that these are related in the first case to chromosomal RNA activity and in the second case to nucleolar RNA activity. The radioactivity is clumped. It does not look like something which is going out of the nucleus very fast, but something very massive. I am talking only of high levels of incorporation, otherwise the nuclear sap is normally very clear and has no radioactivity.

Perry: But you only see that when you use inhibitors or some type of drug.

Sirlin: No, one sees it in the normal cell, too. But the only way to discriminate whether it is nucleolar or chromosomal RNA is by canceling one or the other.

Swift: I have two brief questions, the first being for Dr. Penman. As I mentioned in my paper, in the experiments Dr. Stevens has been doing with *Chironomus* (Stevens, Swift, and Adams, *J Cell Biol* 27: 100A, 1965) there seems to be an increase in ribonucleoprotein granules in the nucleoplasm produced by actinomycin. These particles appear to be formed at a specific site on the chromosome (Balbiani rings) and not in the nucleolus. I wondered if in your studies the polydisperse RNA, which I assume is related to the particles we find, possibly showed a decrease in the rate of its transfer to the cytoplasm. You described a blockage in the transfer of nucleolar RNA. Is the polydisperse RNA fraction also affected?

My second question relates to the statement made by Dr. Perry that ribosomal material always left the nucleus in particulate form. As you probably know, many oocyte nuclei are surrounded by nonparticulate diffuse clouds of cytoplasmic protein or ribonucleoprotein material that does not have a particulate structure. This material is associated with the annuli of the nuclear envelope, and appears to be in the process of migration from nucleus to cytoplasm. If you accept the electron microscopic evidence that this component is nonparticulate, then at least in these oocytes, some material apparently leaves the nucleus in a nonparticulate form.

Penman: I certainly have learned a lot at this Conference. I am not sure I understand Dr. Swift's question, except that I gather there is a morphological change in the nucleoplasm after actinomycin. This could very well be related to the polydisperse nuclear RNA. This material, at least from the kinetic studies, never seems to come into the cytoplasm, but only cycles around in the nucleus, and there is a lot of it. It decays rapidly in actinomycin and if it has something to do with structural events or structural integrity of chromatin, it could very easily be tied up with the phenomenon you suggest.

Perry: In response to Dr. Swift's question, I would like to clarify a point about which a small quorum of the panel members has agreed. When we talk about particles we really mean RNP entities. The exact three-dimensional structure is not precisely specified. It is difficult to say that the configuration of nucleolar particles is exactly that of the cytoplasmic ribosomes or their subunits. It is possible that the particles have a different conformation when they are in the nucleolus.

Penman: I think that the conformation can't be too different from mature ribosomes because the sedimentations are the same. And one is apparently able to detect changes in conformation, for example, the shift from 45S to 30S RNA in EDTA when presumably the structure is expanding.

Perry: Yes, but remember my buoyant density studies (Perry, this Symposium). This is a case in point where zonal centrifugation on sucrose gradients exhibited identical behavior, but yet buoyant density measurements detected a difference in structure.

Gall: I want to comment specifically on the oocyte, since Dr. Swift brought it up. The nucleoplasm in this case is something easily distinguishable from the chromosomes and the nucleoli in autoradiographic experiments. One sees that the nuclear sap itself becomes labeled later than either the nucleoli or chromosomes, so that in theory one could be getting a contribution from both of these into the nuclear sap. In the oocyte of the amphibian, the bulk of the nuclear sap radioactivity, after radioactivity gets to that compartment, is in the 30S RNA which I discussed earlier.

Lara: In *Rhynchosciara*, the 45S RNA is transformed into 28S and 16S RNA without an intermediate 35S RNA step. Apparently, from my data, the salivary gland cells are making mainly rRNA, and the rapidly labeled material has a base composition similar to rRNA. Furthermore, the cells do not seem to have a well-formed nucleolus, as Dr. Pavan has pointed out. Dr. Perry, would you comment on this?

Perry: I think that is very germane to the whole point. As we have heard in Dr. Penman's presentation and from my studies with the azaguanine block (Perry, Nat Cancer Inst Monogr 18: 325-340, 1965), it appears that the 45S to 35S RNA transition occurs in the nucleolus. In other words, the morphological structure one sees when observing the nucleolus is comprised, to a large extent, of 60S RNP particles containing the 35S ribosomal precursor RNA. It is possible that in your system the pool of 60S particles is very small and therefore one cannot detect the 35S RNA intermediate. Concomitantly, one doesn't detect a morphologically distinct nucleolus. I find this rather easy to understand.

Vincent: I ask your indulgence to depart from the specific point of ribosome biogenesis to make a general comment on the nucleolus and its relationship to the function of the whole chromosome. It seems that any site along the chromosome which is actively engaged in RNA production tends to accumulate around it large masses of protein, whether it be a nucleolus, a puff, or a lampbrush loop. A significant proportion of these accumulated proteins are neutral or acidic proteins. I would like to suggest as a generalization, that the mass of the protein material which makes up the nucleolus, and which is not ribosomal protein, is similar to the puff proteins, and that it may be part of a general mechanism involved in the production of all RNA.

Mandel: My first remark concerns the production of 18S and 28S RNA from 45S RNA in cells in a steady state, such as liver cells. If after a short pulse we inhibit the production of 45S RNA by actinomycin, we find labeled 18S and 28S rRNA in the cytoplasm about 4 hours after injection of the precursor. Since with this dosage of actinomycin there is no more synthesis of 45S RNA, we can say that in liver cells, as in HeLa

cells, 28S and 18S rRNA are produced from 45S RNA (Bull Soc Chim Biol, in press). My second remark indicates that we have to be very cautious when talking about a release of ribosomes from nuclei. There is no definite evidence to indicate whether the particles which leave the nuclei are completely formed ribosomes or are particles that are ribosome precursors.

Siebert: I wish to remark briefly about the transition of 45S RNA into any lower class. If one assumes the S value represents molecular size, *i.e.*, the number of nucleotide units, one can calculate that 2 or 3 diester bonds would have to be split for a transition from 45S to 35S RNA. Since there is something between 5,000 and 8,000 diester bonds in a 45S RNA molecule, what could determine the high specificity of the cleavage of much less than 1% of the diester bonds in this molecule?

Perry: I would say that only one cleavage is really needed. One hydrolysis could produce the 35S component. One could conceive of secondary structure or some other steric factor which could determine the specificity. Does anyone on the panel want to make a comment on this? It is a good question, but I have little to add in the way of concrete fact.

Penman: A very brief comment on the cleavage. Of course the cleavage isn't on naked RNA just floating around. I mean an enzyme cleaving that specifically would have to be a very clever enzyme indeed. But the 45S RNA is presumably in the form of RNP and perhaps the protein associated with this initial RNA helps determine the site of cleavage.

Perry: You are implying that the 45S RNA is part of an RNP structure.

Comb: Dr. Penman, you say that the 45S precursor to rRNA is completely methylated. This is of interest since if this molecule must be cleaved to 35S and 16S RNA pieces, one wonders what provides the specificity for such a specific cleavage. One possibility is that the methyl groups act as specific signals for a nuclease as to the site of cleavage.

Perry: A methyl group on the 45S RNA?

Comb: That's right, for certain nucleases.

Feinendegen: I would like to extend Dr. Taylor's question and ask whether there is any information available on ribosome synthesis during various phases of the cell cycle? We heard from Dr. Kasten, for example, that RNA precursor incorporation into the nucleolus is reduced in the beginning of the mid-S phase in his system. We have discussed whether there could be a kind of rebound after this interruption of RNA synthesis, and presumably ribosome formation, in the nucleolus at the S phase. Is there any more information available on that?

Perry: I know Dr. Vincent has some.

Vincent: Our data (unpublished) from synchronous yeast cultures show a burst of ribosome synthesis occurring in the latter part of the cell cycle. The yeast cell has a long G2 and the burst of ribosome synthesis occurs in the very last part of G2. There is ribosome synthesis at a constant, though low, level throughout the entire cell cycle, but the burst of ribosome synthesis occurs at approximately the end of G2.

Perry: I can also add something to that. We have studied the rate of synthesis of nucleolar RNA in cultures of mammalian cells, following the cell cycle by means of time-lapse cinematography (Johnson, Freed, and Perry, unpublished data). This is a very tedious and arduous task in which one makes a motion-picture record of a growing group of cells for about one cell generation and then makes an autoradiograph after a pulse of labeled RNA precursor. One uses the film to ascertain the age of the cells and the autoradiograph to measure rates of incorporation into RNA. In our experiments we did this for over 200 cells. The statistics were not the best in the world, but the general pattern seemed to be clear. There is a rather constant rate of nucleolar incorporation during the first half of the cell cycle and then, probably about mid-S phase, certainly well beyond the beginning of the S phase, there is an increase in the rate to a level which was about 1.6 times the initial rate. We never observed a doubling of the rate of nucleolar incorporation, even though at the end of S phase the number of nucleolar genes is obviously double that at the beginning.

Lewis: We will have to close this panel meeting at this particular point since our time has run out. I am sure I can speak for all the audience in thanking the panel very much for a most stimulating discussion.

The Nucleolus—Retrospect and Prospect¹

C. H. WADDINGTON, *Institute of Animal Genetics, University of Edinburgh, Edinburgh, Scotland*

SUMMARY

This Symposium—summarized under the headings: nucleolar DNA; RNA in the nucleolus; proteins; structure of the nucleolus; and general conclusions—emphasizes the dynamic character of the

nucleolus as an organelle through which materials flow rather than a body of definite basic composition at which a single process is carried out.—*Nat Cancer Inst Monogr* 23: 563–572, 1967.

ALL WHO have attended this Symposium, and all who will later read the published proceedings, will undoubtedly agree that summarizing the data and discussions presented is by no means easy. Possibly, many of us thought when we came here that we had the nucleolus pretty well under control, and that a general consensus would emerge, presenting the activities, functions, and structure of nucleoli in a reasonably orderly and yet comprehensive form. Few of us can still suffer from such optimistic delusions. There is scarcely any point of detail in general agreement, and there are still considerable differences of opinion even on matter of general principle.

I should like to suggest that the basic reason for many of these difficulties is we are only just realizing that the nucleolus is an organelle through which materials are continuously flowing. Its existence and constitution are much more dynamic than, for instance, that of the nucleus as a whole, which is basically characterized as containing the genetic information encoded in DNA, or that of a mitochondrion, fundamentally an organelle in which certain enzymes are arranged in an orderly manner.

Even agreement on a general definition of the nucleolus has been difficult. The word was originally used for relatively large lumps of ribonucleoprotein, visible with the light microscope and associated with recognizable regions on the chromosomes, referred to as nucleolar organizers. However, several different kinds of RNP lumps fulfill these con-

¹ Presented at the International Symposium on The Nucleolus—Its Structure and Function, Montevideo, Uruguay, December 5–10, 1965.

ditions, and the question arises which of these should be considered as true nucleoli and which as merely "nucleolar-like bodies." Considerable clarification of this matter has been produced by recent studies with the technique of molecular hybridization. These have been perhaps the most definite and clear-cut results reported at this Symposium. It has been shown, both in *Drosophila* and *Xenopus*, that the deletion of a chromosomal segment produces simultaneously three different effects: 1) absence of DNA cistrons capable of annealing with ribosomal RNA; 2) failure of ribosomal RNA synthesis; 3) absence of the main nucleolus. This demonstrates unequivocally that the main nucleolus in these forms is concerned with the synthesis of ribosomal RNA.

In the light of these experiments it seems useful to restrict the use of the word *nucleolus* (unqualified) to intranuclear RNP lumps originating from definite places on the chromosomes and concerned with ribosomal RNA synthesis. Only in comparatively few forms do we have a definite proof that bodies commonly referred to as nucleoli fulfill the last part of this definition. Usually this is only an assumption, though probably often fairly safe. However, a definition of this kind makes possible a distinction, becoming more and more necessary, between what are thought to be true nucleoli and other RNP bodies, such as those seen at puffs, or other active sites in chromosomes, which are believed to be unconcerned in the synthesis of ribosomal material. Apparently, we have not agreed on a definite terminology for such bodies, though names such as "nucleoloids" or "nucleolar-like bodies" have been suggested.

In the remaining part of this summary I shall be concerned mainly with true nucleoli as defined above.

COMPOSITION OF NUCLEOLI

It is generally agreed that nucleoli contain three major constituents: proteins making up the greater part of the mass, a considerable quantity of RNA, and a lesser quantity of DNA. I shall consider each of these constituents, taking them in the reverse order.

Nucleolar DNA

The molecular hybridization experiments with the nucleolarless mutants of *Drosophila* and *Xenopus* have shown that in these two forms a single region of the genome contains the DNA cistrons coding for both the 28S and 18S ribosomal RNA. Further, different cistrons have been demonstrated for these two types of RNA, which do not compete with one another in annealing experiments. The number of the DNA cistrons is rather surprisingly large—2,000/3,000 in the amphibia, 400/600 in the insect per diploid set.

The control of the activity of these cistrons is obviously most interesting. In both the forms investigated, the heterozygous nucleus, containing only

one set of DNA ribosomal cistrons instead of two, nevertheless succeeds in producing a normal quantity of ribosomes by some process of regulation. The kinetics of this regulation, and still more its chemical control, obviously deserve, and will presumably shortly receive, much study. There is even some suggestion that in *Drosophila* individuals with less than a full haploid quota of DNA-ribosomal cistrons may develop with a normal phenotype, and probably have the normal complement of ribosomes. They can be detected by breeding tests as subthreshold mutants ("isoalleles") of the *bobbed* locus.

The recent results immediately raise several other general problems:

1) What is the arrangement of ribosomal cistrons in organisms in which nucleoli arise at more than one place in the haploid genome? Shall we discover cases in which the 28S and 18S cistrons are located in different regions of the chromosomes?

2) This raises the question of the relative positions of these two types of cistrons in *Drosophila* and *Xenopus*. Are all the 28S cistrons grouped in one stretch of chromosome and all the 18S ones assembled into the adjacent stretch? Or are they intermingled, perhaps in orderly alternations? There is still no very clear-cut evidence, but perhaps the existence of partial deletions, detected as *bobbed* mutations in *Drosophila*, suggests an intermingling or alternation rather than clear-cut separation of the two types.

3) Do the DNA ribosomal cistrons lie in immediate contact with the nucleolar organizer? It has been pointed out that if, for any reason, a nucleolus fails to form, the secondary constrictions by which the nucleolar organizer is microscopically recognized will usually fail to appear. That a deletion, which removes the nucleolus, also brings about the disappearance of a secondary constriction cannot, therefore, be interpreted as proof that the deletion is located in the region of the constriction. However, in *Drosophila* there is clear-cut genetic evidence that the ribosomal cistrons and the nucleolar organizer are very close to one another, since they both lie between the scute 4 and scute 8 breakage points. Indeed, the cistrons must lie in the region of the *bobbed* locus which has been mapped (1) even nearer to the nucleolar organizer than these two breaks. In *Xenopus* there is no genetic evidence of the location of the deletion removing the ribosomal cistrons. However, other microscopic evidence very strongly suggests that the cistrons lie in the immediate neighborhood of the organizer. This is true in a haplo-deficient organism in which one nucleolus appears at the right region in the normal chromosome, whereas no nucleolus appears on the deficient chromosome. This is easily explained if the cistrons lie near the organizers so that the deficiency has removed them both. However, if one supposed that the cistrons lie at a distance from the organizer, so that the deficiency has left the latter intact, one would have to make rather elaborate secondary hypotheses to explain away the absence of any nucleolus at this remaining nucleolar organizer. If the appearance of nucleolar material at the site of the organizer normally

involves movement from the location of the cistrons, then the only way of accounting for its absence at the organizer of the deficient chromosome would seem to be that this movement must always take place along a chromosome, which would imply that the cistrons are located on the same chromosome as the nuclear organizer, but some distance from it. In the absence of definite genetic proof to the contrary, this might conceivably be the case in *Xenopus*, but the elaborate hypothesis should only be resorted to when there is definite evidence against the view that the situation in the amphibia is similar to that in *Drosophila*.

4) One may ask the question whether the nucleolar organizer has any separate function of its own, or whether it is merely the name given, from the point of view of light-microscopical cytology, to the region of the DNA ribosomal cistrons. In the first place, the organizer is not a single unit. It can be divided by a chromosome break, and the two portions are capable of functioning as organizers when translocated into different positions within the genome. In this it resembles the aggregation of cistrons, which is of course also composite.

If the organizer has a function different from that of the cistrons, *i.e.*, other than that of coding for ribosomal RNA, what is this function? It often has been suggested that the organizer acts as a collecting center, to which materials originating in other parts of the chromosome move. We have seen the difficulties, exhibited in haplo-deficient cells, of applying this argument to the movement of RNA from other parts of the chromosomes. We still need to consider whether there is movement of nucleolar protein into the nucleolus from other parts of the cell, either chromosomes or cytoplasm. Unfortunately, there is little definite evidence as to where in the chromosomes the nucleolar protein originates. The deficiencies removing the DNA cistrons for nucleolar RNA also bring about a failure of accumulation of nucleolar protein at the normal site. It is by no means proved, however, that the deficiencies also have removed the DNA cistrons coding for this protein. In fact, in dying cells of homozygous-deficient *Xenopus*, scattered RNP "blobs" appear on the chromosomes. The nature of these is obscure. They *might* contain nucleolar protein which would normally move to the nucleolar organizer region if it were acting properly, or they *might* be abnormal formations resulting from the removal of competition by the nucleolus for the available supply of nucleotides. Other points to be borne in mind are: (a) the evidence, which goes back a long time [*e.g.*, Navaschin (2)], of competition between organizers of different "strengths" in species hybrids, for instance in *Crepis*. This could, however, be interpreted in terms of the control of the activity of ribosomal RNA cistrons rather than competition in collecting ability between nucleolar organizers; (b) the existence of certain forms [*e.g.*, among chironomids of the genus *Bradysia* studied in our laboratory by Jacob (3)] in which nucleolar material appears at many locations within the chromosomes and never concentrates into a single large nucleolus. Apparently, this situation involves both a scattering of the ribosomal cistrons through-

out the genome, in contrast to the bunching together of all such cistrons in *Drosophila* and *Xenopus*, and also an absence of a nucleolar organizer in the sense of an active assembly point.

The simplest hypothesis is to regard the "nucleolar organizer" as another name for the stretch of DNA coding for ribosomal RNA, but to suppose that when these cistrons are actually producing the corresponding RNAs, nucleolar proteins, possibly originating in other parts of the chromosomes, may accumulate around the active region.

5) What is the relation between the ribosomal RNA cistrons and the DNA occurring within the nucleolus?

There is increasing evidence, particularly from electron microscopy, of the occurrence of considerable amounts of DNA within the nucleolus. Apart from the evidence presented in this Symposium, Dr. J. Jacob (4) in our laboratory at Edinburgh has good histochemical electron microscopic evidence for the existence of this constituent. DNA appears to exist in the nucleolus in the form of a tangled filament. Is there any good reason to reject the simplest hypothesis, namely, that this is the stretch of DNA ribosomal-RNA cistrons we have been considering? A quantitative point is of interest. If the ribosomal cistrons of *Xenopus* were stretched out in a simple double helix, it would be several hundred μ long, possibly even as much as a millimeter. This must be accommodated somewhere. It would seem a simple and sensible arrangement to pack it inside the nucleolus, where it might be expected to produce appearances very much like those which have actually been seen.

RNA in the Nucleolus

The DNA cistrons, whose presence is necessary for the appearance of the nucleolus, code separately for the 28 and 18S ribosomal RNA. However, labeling experiments show that the first type of RNA to be labeled in the nucleolus belongs to neither of these species, but has a sedimentation rate of about 45S. It seems to be generally agreed that at least some of this 45S RNA is a precursor of the 28S material, the synthetic pathway probably passing through a 30–35S intermediate stage. The 28S material is, of course, eventually passed into this cytoplasm. Although some intriguing data were presented about this, the exact mechanism of the transport still remains obscure.

There is less agreement about the appearance of the 18S ribosomal RNA. Investigations of the RNAs of isolated nucleoli usually reveal little 18S material, and some authors doubt whether it is synthesized in that organelle at all. However, when labeled nucleotides are offered to cells, radioactivity very rapidly appears in cytoplasmic 18S RNA. As we have seen above, there is little doubt that the DNA cistrons coding for the 18S RNA are present in the nucleolar organizer region. The suggestion that the 18S material is actually synthesized in the cytoplasm requires transporting the information from the chromosomes to the site of synthesis, and no suggestions have been made as to how this can be done. The

alternative is that the synthesis actually takes place in the nucleolus, but the 18S material moves into the cytoplasm in a much shorter time after its synthesis than does the 28S, so that the nucleolar pool of 18S is always very small.

In any case we have to inquire whether the 18S, like the 28S RNA, passes through a number of precursor stages before attaining its final form. If the synthesis takes place in the nucleolus, a precursor might well be sought among the 45S rapidly labeled RNA. It is rather unlikely, however, that a single 45S species of RNA breaks down to give both 28S and 18S end products. If there were such a single large precursor molecule, this would first seem to imply that the 28 and 18S DNA cistrons alternated with one another, so that short, 2, 3, or 4 unit polycistrons contain both kinds. It is, however, difficult to see how a single 45S RNA particle could break up into an equal number of 28 and 18S units, the proportions these two species of RNA have in the mature cell. One may, however, explore the possibility that the 18S precursor is different from the precursor of the 28S RNA. This would not preclude the possibility that it would also sediment at 45S, since even if the 45S is monotypic in sedimentation rate, it may be polytypic in chemical specificity. Indeed, some speakers have suggested a third type of 45S RNA, DNA-like in composition.

Obviously, from these abbreviated remarks, there are still almost more questions than answers concerning the nature and the location of the synthetic sequences by which the final ribosomal 28S and 18S RNAs come into being. The main point emerging in this connection is that the nucleolus is only, as it were, a passageway through which RNAs are moving more or less rapidly, being processed as they go through. We need much more information about the kinetics of these processes. Presumably the over-all rate will depend on the metabolic condition of the cell, for instance, whether it is accumulating ribosomes, or losing them, or maintaining a stable population. Even in stable mature cells, supposedly in the last category, the rate of synthesis of new ribosomes will have to be adjusted in accordance with the average half-life of a ribosome, and there are some indications that this varies in different cell types. The sizes of the pools of the various constituents which may be expected to exist in the nucleolus are therefore bound to depend on a rather complicated balance of rates of flow. If the relative rates of the various contributing processes of synthesis and transport can also be altered, the situation becomes even more complicated. It will take some time and probably a good deal of debate before the labeling experiments can be interpreted in the light of all these considerations.

It is rather surprising that cells containing DNA cistrons capable of annealing with 28 and 18S RNA from fully formed ribosomes have not, during the course of evolution, found some simpler way of producing these RNAs than the apparently complicated rigmarole of going through 45S, 30-35S, and possibly other intermediates. This may be connected

with the fact that ribosomes must have a highly complex tertiary or quaternary structure, which makes them capable, on the one hand, of moving along a strand of messenger RNA, and, on the other, of accepting transfer RNAs in such a way as to bring their attached amino acids into position to link up to form polypeptide chains. On the fact of it, the cell has a difficult task to assemble a machine tool of such characteristics, but we shall not fully understand the RNA metabolism of the nucleolus until we have some idea why the assembling goes through the particular precursor stages that seem to be involved. Indeed, the general nature of these precursors is still obscure. I know of no reports of 45S RNAs in the nucleoli of plants, nor do materials of this character show up in labeling experiments with bacteria. It may be that their appearance in animal nucleoli is due, not to a sequence of transformation stages of the RNA, but rather to the formation of some sort of relatively stable aggregate between normal ribosomal RNA and other components of the cell (perhaps glycogen?).

We might expect still other types of RNA in the nucleolus. Since the organelle contains a great deal of protein, we would expect to find much DNA-like messenger RNA coding for this protein. There has been some mention of a DNA-like 45S RNA, but this seems to be more characteristic of the nucleoplasm than of the nucleolus itself. We seem to have little information about messenger-like RNA within the nucleolus.

The appearance of transfer RNA in the nucleolus has also been discussed, with as yet no agreement on this score. We have been presented with evidence that RNAs sedimenting at 4S are synthesized in the nucleolus in nongrowing larval salivary glands of a chironomid. There is some chemical evidence (methylation) that this RNA is transfer RNA, but the final demonstration that it can be coupled to amino acids by activating enzymes is still not available. However, in a quite different type of tissue (growing *Vicia faba* meristems) a procedure which seems specifically to extract transfer RNA did not lead to any lowering of the autoradiographic counts over the nucleoli of labeled cells. But the degree to which one would expect these counts to be lowered would depend on the proportion which the transfer RNA, if present, bears to the other RNA constituents of the organelle, and there do not yet seem to be any data from which this can be properly estimated. We are here again faced with the question of pool sizes and the rates at which synthesized materials accumulate in or leave the nucleolus.

Proteins

We were forcefully reminded again, at this Symposium, that most (80–90%) of the dry matter of the nucleolus consists of protein. Unfortunately we still have very inadequate insights into the natural functions of this material. When proteins extracted from isolated nucleoli are analyzed, at least half, or even more, have a different constitution to

ribosomal protein. However, in some instances a considerable fraction of "residual protein" is ribosome-like, and even in starfish oocyte nucleoli, where the main bulk is unlike ribosomal protein, a certain fraction has the same solubility characteristics and is immunologically indistinguishable from it. This may well be a pool of ribosomal protein, which may have been actually synthesized in the nucleolus, and which is in transit through that structure like the ribosomal RNAs.

The nature of the nonribosomal nucleolar proteins remains most mysterious. All can scarcely be polymerases or other enzymatically active proteins, though these have been shown to be present. Perhaps one might suspect some relationship with the protein which becomes associated with RNA in puffed regions of the chromosomes. Such a relationship might account for some of the phenomena suggesting the existence of a competition between the development of nucleoli and of puffs. Again one might suspect that some of the nucleolar proteins help in stabilizing some of the species of RNAs by forming complexes somewhat similar to the messenger-RNA-protein bodies which Nemer and Spirin (5, 6) have named "informosomes." Finally, some of them may play a role in the synthesis of ribosomal proteins comparable to that which the ribosomal proteins themselves have in relation to other proteins.

The whole problem of the synthesis of ribosomal proteins seems at present to be very obscure. Can we suppose that the 28S and 18S ribosomal RNAs, or their precursors, are themselves the messenger RNAs carrying information for the synthesis of the ribosomal proteins? If not, what are the corresponding messengers, and where in the genome are they produced? In bacteria they are known, in some instances, to lie near the ribosomal RNA cistrons, and the soluble-RNA cistrons as well, but we have no information about their location in any eukaryotic cells. Again, does the synthesis of the ribosomal protein involve the participation of a preexisting ribosome, or are they formed by a rather different type of cellular machine tool, perhaps involving the particulate component of the nucleolus composed of RNP units somewhat smaller than the cytoplasmic ribosomes? Even after this Symposium, the whole of this area does not seem to have emerged as anything more definite than a tempting arena for speculations.

STRUCTURE OF THE NUCLEOUS

During the Symposium a large number of structural components of the nucleolus were mentioned. I think one needs to consider at least 8, some of which, however, may be different names for the same structures.

1) *A particulate component.*—This appears under electron microscopy as a mass of particles, each somewhat smaller than a cytoplasmic ribosome. They are certainly ribonucleoprotein in composition, and probably their RNA is the 28S ribosomal RNA material which in normal nucleoli exists as RNP.

2) *A fibrillar component*.—Something similar may be found in the nucleolus-like bodies in nucleolarless cells shortly before their death, but these may be aggregates of proteins rather than of RNP fibers. In the normal nucleolus the particulate and fibrillar materials are often intermingled in rather coarse masses and they tend to become more definitely segregated from one another under the influence of actinomycin D.

3) *An amorphous component*.—This was probably mainly protein.

4) *DNA*.—By autoradiography this can be detected as diffusely scattered around the interior of the nucleolus. The more definite evidence from the electron microscope suggests that it forms a tangled network, probably connected to the nucleolar organizer, but sometimes giving rise to strands lying near the cortex of the nucleolus. Probably this material is the stretch of chromosome containing the DNA ribosomal RNA cistrons, as suggested above.

5) *The nucleolonema*.—This word has been used by light microscopists as a name for the tangled threadlike bodies which may be revealed within the nucleolus by certain fixing and staining procedures. Its relation to the DNA threads just mentioned remains obscure. Histochemical evidence suggests the nucleolonema predominantly composed of RNP. However, the DNA chromosomal cistrons are presumably actively engaged in the production of corresponding RNAs, and it is quite tempting to suppose that the nucleolonema consists of a core of DNA around which RNA and protein have accumulated, but this has perhaps not yet been definitely demonstrated.

6) *Vacuoles*.—Nothing much seems to be known about their function.

7) *Nucleolus-associated chromatin*.—Material of a DNA-histone nature is often found around the external periphery of the nucleolus. Possibly this represents the DNA-ribosomal cistrons, but if we suppose that these penetrate into the depth of the nucleolus, as suggested above, we should have to suppose that the nucleolus-associated chromosome in the present sense represents something else, perhaps the cistrons for nucleolar and ribosomal proteins, possibly merely a random assortment of chromosomal material, by chance precipitated onto the nucleolar surface.

8) *Perinucleolar structures*.—In some types of cells the nucleolus has been described as being enclosed within specialized structures much more massive than the nucleolus-associated chromatin. Both the materials under these last two headings need, of course, special attention from procedures designed for the isolation of pure nucleolar material in bulk from homogenized cells or nuclei.

As might be expected, types of cells vary considerably in the ways in which these various structures are mutually arranged and in the degree to which the different constituents are developed in different metabolic situations. In general, it will probably be impossible to make very much sense out of the various microscopical images until they can be interpreted in terms of the synthetic processes in which the various elements in the nucleolar structure are engaged.

GENERAL CONCLUSIONS

The general conclusion which suggests itself, at least to me, after listening to this Symposium is as follows: The nucleolus probably should not be considered a relatively simple organelle with a single function, comparable to a machine tool turning out a particular part of an automobile. It is not just "the organelle where the cell manufactures ribosomes." It is rather a structure through which materials of several different kinds are flowing, comparable more to a whole production line than to a single machine tool. The greatest need in the immediate future seems to be further information about the rates of the various flows contributing to this production line and of the individual steps within each flow, by which, for instance, several types of RNAs, and presumably of proteins also, are processed through a number of stages from one precursor to another until they finally leave the nucleolus and are transferred through the nucleoplasm into the cytoplasm. The timing of these various transit times and flow rates will be difficult, but apparently is the indispensable next step in our understanding.

RESUMEN

Resumen del simposio. Los títulos: ADN nucleolar; ARN en el nucleolo; proteínas; estructura del nucleolo; y conclusiones generales; que enfatizan su carácter dinámico, como un organelo a través del cual fluyen los materiales mas bien que un cuerpo de composición básica definida en el cual se lleva a cabo un único proceso.

REFERENCES

- (1) COOPER, K. W.: Cytogenetic analysis of major heterochromatic elements (especially Xh and Y) in *Drosophila melanogaster*, and the theory of "heterochromatin." *Chromosoma* 10: 535-588, 1959.
- (2) NAVASCHIN, M.: Chromosome alterations caused by hybridisation and their bearing upon certain general genetic problems. *Cytologia* 5: 169-203, 1934.
- (3) JACOB, J., and SIRLIN, J. L.: Electron microscope studies on salivary gland cells. I. The nucleus of *Bradysia mycorum* Frey (Sciaridae), with special reference to the nucleolus. *J Cell Biol* 17: 153-165, 1963.
- (4) JACOB, J.: Personal communication. In press, *Nature*, 1965.
- (5) NEMER, M., and INFANTE, A.: Informosomes: Messenger RNA-protein complexes. *Fed Proc* 24: 283, 1965.
- (6) SPIRIN, A. S., BELITSINA, N. V., and AITKHOZHIN, M. A.: Informational RNA in early embryogenesis. *Zh Obschch Biol* 25: 321-338, 1964.

APPENDIX

Prior to the Montevideo Symposium on the nucleolus, Dr. Hollaender, as President of the Symposium, asked Dr. Hewson Swift to chair and select a committee to present to the Symposium recommendations for standardizing the nomenclature of the nucleolus. This committee met during the week of the Symposium in Montevideo, and its report was presented to the Symposium during the final session. It was moved, seconded, and unanimously approved by the Symposium participants that the committee report be accepted and included in the proceedings of the Symposium.

REPORT OF THE NUCLEOLUS NOMENCLATURE COMMITTEE

It has been suggested that the Symposium agree, if possible, to a statement concerning nucleolar nomenclature.

Any statement on the nomenclature of the nucleolus made at this time must of necessity be incomplete, and subject to revision with increased knowledge. Although the following statement may be applicable to the nucleoli of many cell types, there is a possibility that other special cells occur to which it may not apply.

The nucleolus is a body largely containing protein and RNA, often compact, but in some cells diffuse or fragmented, which arises in connection with one (occasionally more) specific chromosome site in the haploid karyotype. We may call this site the *nucleolus organizer*, but without implication as to whether this is specifically a site of collection or of synthesis. The organizer site is usually marked in metaphase chromosomes by a *secondary constriction* when a nucleolus is present in the preceding prophase.

The organizer region probably contains the *cistrons for ribosomal RNA*. The possibility that it contains the cistrons for other RNA fractions cannot yet be eliminated.

Nucleoli of most cell types probably remain attached to the organizer site in interphase. Nucleoli are thus in close contact with the DNA and histone of the organizer. Other DNA-histone regions may in varying degree penetrate and interdigitate with the nucleolar substance, forming the *nucleolus associated chromatin*. Some of this chromatin may be unrelated to nucleolar function.

The RNA of the nucleolus contains the precursors of at least some ribosomal RNA, but possibly not ribosomal RNA itself. It may also contain nonribosomal RNA.

The predominant component of the nucleolus is protein. Several different kinds of proteins are present. Whether or not these include ribosomal proteins is not yet clear.

Nucleoli viewed under the electron microscope contain a *fibrillar component*, about 50 to 80 Å in width, and a *particulate component* about 150 Å in diameter. Both of these components are composed of ribonucleoproteins. The particulate component somewhat resembles cytoplasmic ribosomes, but is usually smaller and less regular

in outline. Many nucleoli possess regions which are predominantly fibrillar or predominantly particulate. In some nucleoli, the fibrillar material possesses a central and the particulate component a peripheral localization. Nucleoli have no limiting membrane.

Nucleoli frequently contain threadlike elements about 0.1μ in diameter, or larger, which may be called *nucleolonemata*. These often appear irregular and anastomosing, arranged either loosely or in a compact mass. They may be composed of either or both fibrillar or particulate components, and thus consist predominantly of ribonucleoprotein. The use of the term "nucleolonema" is restricted here to this sense, and the relationship, if any, between the nucleolonemal strands and the chromosomes is presently not clear.

Nucleoli frequently contain more or less spherical inclusions, usually of lower density than the surrounding nucleolar mass, which may be called *nucleolar vacuoles*. They possess no limiting membrane, and have variable contents depending on the type and condition of the cell. Some vacuoles contain no visible formed elements. Others contain proteins in variable concentrations, scattered ribosomes, and other constituents. Nucleolar vacuoles sometimes appear adjacent to penetrating portions of chromatin.

The general terms we have applied to nucleoli and related chromosomal regions are as follows:

Chromosomal components	Organizer
	Cistron for ribosomal RNA
	Secondary constriction
	Nucleolus associated chromatin
Nucleolar substance	Fibrillar component
	Particulate component
	Nucleolonema
	Vacuoles

Other structures may occur within nucleoli which cannot be related to the above components. At present most of these are poorly understood, and it seems unwise to name them at this time.

Ribonucleoprotein inclusions somewhat similar to nucleoli in size and shape, but not forming at the nucleolus organizer, often occur within nuclei. We do not believe these structures should be called nucleoli unless their ability functionally to replace the true nucleolus can be established.

COMMITTEE ON NUCLEOLAR NOMENCLATURE
CLEMENTE ESTABLE (*Honorary Chairman*)

WILHELM BERNHARD

JOSEPH GALL

ROBERT PERRY

JULIO SIRLIN

HEWSON SWIFT (*Chairman*)

PARTICIPANTS

Argentina

- Andrieu, Ana, *Facultad de Química y Farmacia, Universidad Nacional de la Plata*
 Bard, Enzo, *Facultad de Química y Farmacia, Universidad Nacional de la Plata*
 Bianchi, Martha de, *Comisión de Investigación Científica, La Plata*
 Bianchi, N. O., *Comisión de Investigación Científica, La Plata*
 Boggiano, A. J., *Facultad de Agronomía de Tucumán*
 Casanello, Delia, *Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires*
 Diez, Julio, *Laboratorio de Genética, Universidad de Buenos Aires*
 Doria, Irma, *Facultad de Química y Farmacia, Universidad Nacional de La Plata*
 Dos Reis, Maria, *Instituto Biológico de La Plata*
 Dulout, F. N., *Facultad de Agronomía y Zootécnica, Universidad de Tucumán*
 Favelukes, Gabriel, *Facultad de Química y Farmacia, Universidad Nacional de La Plata*
 Favelukes, Susana, *Facultad de Medicina, Universidad de Buenos Aires*
 Ferreyra, M. E., *Laboratorio de Genética, Universidad de Buenos Aires*
 Grau, Oscar, *Facultad de Química y Farmacia, Universidad Nacional de La Plata*
 Lapetina, E. G., *Instituto de Anatomía General y Embriología, Universidad de Buenos Aires*
 La Torre, J. L., *Instituto de Anatomía General y Embriología, Universidad de Buenos Aires*
 Lombardo, Héctor, *Instituto Fiebre Aftosa, Universidad de Buenos Aires*
 Mariano, Enrique, *Facultad de Agronomía y Veterinaria, Universidad de Buenos Aires*
 Maroni, Gustavo, *Laboratorio de Genética, Universidad de Buenos Aires*
 Mazar-Barnett, Beatriz, *Comisión Nacional de Energía Atómica, Buenos Aires*
 Moreira, J. L., *Comisión Nacional de Energía Atómica, Buenos Aires*
 Muñoz, E. R., *Comisión Nacional de Energía Atómica, Buenos Aires*
 Pavlovsky, Alfredo, *Instituto de Investigaciones Hematológicas, Universidad de Buenos Aires*
 Reader, T. A., *Instituto de Anatomía General y Embriología, Universidad de Buenos Aires*
 Reisin, Ignacio, *Facultad de Agronomía y Veterinaria, Universidad de Buenos Aires*
 Reissig, J. L., *Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires*
 Roosiger,
 Rossi, F. M., *Facultad de Agronomía y Veterinaria, Universidad de Buenos Aires*
 Sorgentini, Delia, *Facultad de Química y Farmacia, Universidad Nacional de La Plata*
 Spitz, Lidia de, *Facultad de Medicina, Universidad Nacional de La Plata*
 Spitz, Moisés, *Facultad de Química, Universidad Nacional de la Plata*
 Tandler, C. J., *Instituto de Anatomía General y Embriología, Universidad de Buenos Aires*
 Valencia, Hélida Dopchiz de, *Facultad de Química y Veterinaria, Universidad Nacional de La Plata*
 Valencia, J. I., *Laboratorio de Genética, Universidad de Buenos Aires*
 Valencia, Ruby M. de, *Comisión Nacional de Energía Atómica, Buenos Aires*

Vasquez, César, *Instituto de Investigaciones Hematológicas, Universidad de Buenos Aires*
 Vonech, Eugenio, *Facultad de Agronomía y Veterinaria, Universidad de Buenos Aires*
 Weisman, Roberto, *Laboratorio de Genética, Universidad de Buenos Aires*
 Zylber, Ester, *Facultad de Química y Farmacia, Universidad Nacional de La Plata*

Belgium

Ficq, Adrienne, *Laboratoire de Morphologie animale, Université Libre de Bruxelles*

Brazil

Blumenschein, Almiro, *Escola Superior Agricultura "Luiz de Queiroz," Universidade de São Paulo, Piracicaba*
 Brentani, Mitze, *Faculdade de Medicina, Universidade de São Paulo*
 Brentani, Ricardo, *Faculdade de Medicina, Universidade de São Paulo*
 Lara, F. J. S., *Laboratório de Bioquímica, Universidade de São Paulo*
 Pavan, Crodowaldo, *Departamento de Biologia Geral, Universidade de São Paulo*
 Sauaia, Heni, *Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo*
 Schreiber, Giorgio, *Instituto de Biologia, Universidade de Minas Gerais*

Canada

Chouinard, L. A., *Department of Anatomy, McGill University, Montreal*
 *Lafontaine, J. G., *Faculty of Sciences, Laval University, Quebec*

Chile

Basilio, Carlos, *Instituto de Química Fisiológica y Patológica, Universidad de Chile, Santiago*
 Cardenas, Maria de la Luz, *Facultad de Veterinaria, Universidad de Chile, Santiago*
 Coronado, Alfonso, *Instituto de Química Fisiológica y Patológica, Universidad de Chile, Santiago*
 Fischer, Siegmund, *Escuela de Medicina, Universidad de Chile, Santiago*
 Grado, Carmen, *Escuela de Medicina, Universidad de Chile, Santiago*
 Hoecker, Gustavo, *Facultad de Ciencias, Universidad de Chile, Santiago*
 Izquierdo, Luis, *Facultad de Ciencias, Universidad de Chile, Santiago*
 Mardones, Elvira, *Instituto de Química Fisiológica y Patológica, Escuela de Medicina, Universidad de Chile, Santiago*
 Martinez, Carlos, *Escuela de Medicina, Universidad de Chile, Santiago*
 Muñoz-Fredes, Eliana, *Escuela de Medicina, Universidad de Chile, Santiago*
 Murialdo, Helios, *Facultad de Ciencias, Universidad de Chile, Santiago*
 Pizarro, Olga, *Escuela de Medicina Veterinaria, Universidad de Chile, Santiago*
 Ramos, Alicia, *Escuela de Medicina, Universidad de Chile, Santiago*
 Valladares, Pedro, *Facultad de Ciencias, Universidad de Chile, Santiago*
 Vergara, Juan, *Facultad de Medicina, Universidad de Chile, Santiago*

France

Bernhard, William, *Institut de Recherches Scientifiques sur le Cancer, Université de Paris*

Feinendegen, L. E., *Institut du Radium, Laboratoire Pasteur, Paris*
 Mandel, Paul, *Institut de Chimie Biologique, Faculté de Médecine de Strasbourg*
 Zajdela, Francois, *Institut du Radium, Laboratoire Pasteur, Paris*

Germany

Lettré, Renate, *Institut für experimentelle Krebsforschung, Universität Heidelberg*
 Pelling, Claus, *Max-Planck-Institut für Biologie, Tübingen*
 Siebert, Günther, *Physiologisch-Chemisches Institut der Johannes Gutenberg-Universität, Mainz*

Italy

Maggio, Rachele, *Istituto di Anatomia Comparata, Università di Palermo*
 Ritossa, Ferruccio, *Laboratorio Internazionale di Genetica e Biofisica di Napoli*

Peru

Castro de La Mata, Olga, *Facultad de Medicina, Lima*

Scotland

Birnstiel, Max L., *Institute of Animal Genetics, University of Edinburgh*
 Sirlin, J. L., *Institute of Animal Genetics, University of Edinburgh*
 Waddington, C. H., *Institute of Animal Genetics, University of Edinburgh*

Sweden

*Edström, J. E., *Karolinska Institutet, Stockholm*
 Stenram, Unne, *Department of Pathology, University of Uppsala*

United States

Barr, H. J., *Zoology Research Building, University of Wisconsin, Madison*
 Brown, D. D., *Department of Embryology, Carnegie Institution of Washington, Baltimore, Maryland*
 Busch, Harris, *Department of Pharmacology, Baylor University College of Medicine Houston, Texas*
 Cohen, P. P., *Department of Physiological Chemistry, University of Wisconsin Medical School, Madison*
 Comb, D. G., *Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts*
 Das, N.K., *Department of Zoology, University of California, Berkeley*
 Gall, J. G., *Department of Biology, Yale University, New Haven, Connecticut*
 Hollaender, Alexander, *Biology Division, Oak Ridge National Laboratory, Tennessee*
 Hyde, B. B., *Department of Botany, University of Vermont, Burlington*
 Kasten, F. H., *Ultrastructural Cytochemistry Section, Pasadena Foundation for Medical Research, California*

- Kleinfeld, Ruth G., *Department of Pharmacology, State University of New York, Syracuse*
- Lewis, Herman, *Division of Biological and Medical Sciences, National Science Foundation, Washington, D.C.*
- Love, Robert, *Department of Pathology, Jefferson Medical College, Philadelphia, Pennsylvania*
- McClintock, Barabara, *Genetics Research Unit, Carnegie Institution of Washington, Cold Spring Harbor, Long Island, New York*
- Miller, O. L. Jr., *Biology Division, Oak Ridge National Laboratory, Tennessee*
- Penman, Sheldon, *Department of Biology, Massachusetts Institute of Technology, Cambridge*
- Perry, R. P., *The Institute for Cancer Research, Philadelphia, Pennsylvania*
- Schultz, Jack, *The Institute for Cancer Research, Philadelphia, Pennsylvania*
- Swift, Hewson, *Whitman Laboratory, University of Chicago, Illinois*
- Taylor, J. H., *Institute of Molecular Biophysics, The Florida State University, Tallahassee*
- Totter, J. R., *Division of Biology and Medicine, U.S. Atomic Energy Commission, Washington, D.C.*
- Vincent, W. S., *Department of Anatomy and Cell Biology, School of Medicine, University of Pittsburgh, Pennsylvania*
- von Borstel, R. C., *Biology Division, Oak Ridge National Laboratory, Tennessee*
- Wallace, Hugh, *Biology Department, Amherst College, Massachusetts*
- Woodruff, Nathan, *Scientific Attaché, American Embassy, Buenos Aires, Argentina*
- Woods, P. S., *Department of Biological Sciences, University of Delaware, Newark*

Uruguay

- Appeltauer, Carlos, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Cardoso, Horacio, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Crosa, Orfeo, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Díaz, Manuel, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Drets, Máximo, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Estable, Clemente, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Gerard, Gabriel, *Facultad de Humanidades y Ciencias de Montevideo*
- Korc, Israel, *Facultad de Medicina, Montevideo*
- Pérez Mosquera, Glauca, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Poletti, Heraldó, *Facultad de Medicina, Universidad de la República, Montevideo*
- Rebollo, María Antonieta, *Facultad de Medicina, Montevideo*
- Sáez, F. A., *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Sotelo, J. Roberto, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Wettstein, Rodolfo, *Instituto de Investigación de Ciencias Biológicas, Montevideo*
- Zorrilla, Nadia Brum de, *Instituto de Investigación de Ciencias Biológicas, Montevideo*

USSR

- *Georgiev, G. P., *Institute of Molecular Biology, Academy of Sciences of the USSR, Moscow*

Venezuela

- Patetta Queirolo, Miguel, *Facultad de Medicina, Universidad de Carabobo, Valencia*

*These individuals were unable to attend the Symposium but contributed to the Proceedings.

NAME INDEX

- A**ckermann, W. W., 170, 175
 Acs, G., 376, 378
 Adams, B. J., 153, 153, 156, 558
 Adams, H., 193, 195, 204, 206, 207, 247, 250, 286, 292
 Agrell, I., 114, 118
 Aitkhoshin, M. A., 522, 524, 535, 544, 572
 Albertini, de M. L., 206, 207
 Albuquerque, R. M., 129, 135
 Alexander, J., 5
 Alfert, M., 147, 155, 182, 184, 185, 337, 344, 359, 361
 Allfrey, V. G., 19, 26, 57, 59, 112, 118, 219, 224, 232, 266, 298, 305, 306, 317, 342, 345, 475, 479, 481, 484, 486
 Altmann, H. W., 16, 25, 128, 134, 280, 385
 Amano, M., 20, 27, 153, 156, 371, 377
 Amenta, P. S., 414, 418
 Ames, B. N., 334, 335, 479, 486
 Anan'eva, L. N., 513, 523
 Anderson, J. R., 182, 186
 Anderson, R. M., 370, 377
 Angelov, E., 523, 524
 Arendell, J. P., 193
 Arrighi, F. E., 97, 99
 Ashton, F. T., 9, 89
 Asriyan, I. S., 513, 523
 Astbury, W. T., 5, 7
 Astrachan, L., 229, 233
 Attardi, G., 454, 460, 470, 518, 519, 524
 Atwood, K. C., 59, 399, 449, 459, 470, 471, 543
 Austin, C. R., 302, 307
 Axelrod, A. E., 237, 249

Bäckström, S., 416, 418
 Balbiani, E. G., 167, 174
 Baltimore, D., 21, 27
 Baltus, E., 225, 226, 232, 235, 236, 243, 246, 247, 248, 250, 285, 286, 291, 312, 317
 Bang, F. B., 14, 25, 91, 98, 127, 133
 Bannasch, P., 16, 25
 Barbu, E., 237, 248
 Barr, H. J., 52, 66, 269, 301, 306, 411, 413, 414, 415, 417, 418, 422, 424, 427, 429, 432, 446, 441, 443
 Barski, G., 21, 27
 Barth, L. G., 416, 419
 Barth, L. J., 416, 419
 Barth, P., 18, 26
 Basilio, C., 222, 473
 Bassel, B., 268, 233
 Battips, D. M., 168, 175
 Bauer, A., 14, 15, 25, 127, 133, 269, 408
 Beaudreau, G., 209
 Bechtina, O. G., 302, 307
 Beck, S. J., 143, 182, 186
 Becker, Y., 154, 157, 503, 507, 531, 543
 Beermann, W., 147, 155, 156, 166, 184, 187, 224, 229, 231, 233, 264, 266, 302, 306, 342, 345, 393, 394, 397, 398, 399, 400, 402, 404, 406, 432, 443, 463, 471
 Belar, K., 3
 Belitsina, N. V., 523, 524, 535, 542, 543, 544, 572
 Bellamy, A. D., 518, 524
 Bencosme, S. A., 127, 128, 132, 134, 224, 231
 Berenbaum, M. C., 111, 118
 Bergqvist, H. A., 114, 118
 Bernard, C., 94, 99
 Bernhard, W., 13, 14, 15, 16, 17, 18, 19, 21, 23, 24, 25, 26, 27, 28, 36, 37, 38, 91, 94, 99, 127, 128, 133, 134, 172, 175, 183, 186, 209, 574
 Berzelius, J. J., 5
 Bess, L. G., 360, 361
 Bharadwaj, T. P., 167, 168, 174
 Bhatia, 423
 Bianchi, N. O., 367
 Bieleavsky, N., 312, 317
 Bierling, R., 22, 27
 Bigley, N. J., 237, 249
 Birnstiel, M. L., 39, 41, 43, 45, 57, 59, 66, 68, 70, 126, 133, 183, 184, 186, 187, 210, 214, 215, 219, 222, 224, 225, 226, 227, 228, 232, 246, 247, 249, 251, 255, 256, 257, 258, 261, 263, 264, 265, 266, 269, 271, 277, 278, 283, 284, 286, 292, 301, 306, 327, 335, 337, 342, 344, 396, 397, 399, 422, 423, 426, 427, 428, 429, 432, 433, 434, 436, 441, 443, 444, 445, 446, 450, 457, 460, 463, 470, 471, 472, 507, 508, 515, 520, 523, 524, 527, 528, 543, 548, 549, 550, 551, 554, 555, 557

- Bleyman, M., 461, 471
 Bloch, D. P., 359, 361
 Bloch, M., 390
 Boiron, M., 277, 278
 Bolognari, A., 15, 25
 Bolton, E. T., 434, 444
 Bond, V. P., 378
 Bonner, J., 227, 232, 354, 361
 Borek, E., 226, 232, 271, 272, 278, 292, 427, 429, 470
 Borysko, E., 14, 25, 91, 98, 127, 133
 Brachet, J., 6, 7, 57, 59, 311, 312, 316, 317, 325, 326, 334, 450, 469, 475, 485
 Branda, R., 325, 327, 329, 331, 332, 334
 Brandt, E. E., 286, 292
 Braverman, M. H., 417, 419
 Bray, G. A., 477, 486
 Bridges, C. B., 5
 Brinkley, B. R., 97, 99, 105
 Bristow, D. A., 425, 428
 Britten, R. J., 523
 Brown, C. A., 298, 305
 Brown, D. D., 59, 147, 152, 155, 184, 187, 208, 209, 226, 228, 232, 236, 248, 253, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 311, 312, 316, 317, 327, 335, 336, 396, 399, 406, 411, 415, 416, 417, 422, 425, 426, 429, 432, 436, 441, 442, 443, 444, 445, 447, 450, 459, 469, 473, 475, 476, 477, 478, 484, 485, 486, 487, 549, 550, 551
 Brown, R., 432, 444
 Brown, S. W., 70, 71
 Bruner, R., 477, 486
 Bruns, G. P., 228, 232
 Bucher, N. L. R., 375, 377
 Buffé, D., 22, 27
 Burnham, C. R., 148, 156
 Busch, H., 13, 15, 16, 21, 25, 27, 58, 59, 66, 105, 109, 117, 193, 194, 195, 196, 199, 202, 203, 204, 206, 207, 208, 209, 210, 221, 246, 247, 259, 285, 287, 291, 292, 293, 337, 344, 369, 375, 376, 377, 378, 395, 399, 406, 455, 520, 524
 Byvoet, P., 13, 25, 58, 59, 286, 292, 337, 344
 Cajal, Ramon y., 93, 99, 180
 Callan, H. G., 54, 56, 59, 152, 156, 298, 305, 475, 476, 484, 486
 Canellakis, E. S., 277, 278
 Cape, M., 317
 Carnoy, J. B., 1, 2
 Caro, L. G., 19, 26
 Caspersson, T., 5, 6, 7, 450, 469, 485
 Caston, J. D., 312, 317
 Chalkley, H. W., 241, 249, 370, 377
 Chambon, P., 292
 Chargaff, E., 333, 335
 Chen, T-T., 116, 118
 Chentsov, Y. S., 514, 520, 523
 Chipchase, M. I. H., 41, 45, 210, 217, 219, 226, 227, 232, 246, 247, 249, 251, 337, 342, 344, 432, 441, 444, 450, 460, 470, 515, 520, 523, 524, 530, 543, 550, 554
 Chouinard, L. A., 39, 43, 44, 45, 52, 68, 69, 70, 96, 99, 125, 126, 128, 129, 133, 134, 142, 146, 155, 184, 187, 416, 418
 Clever, U., 153, 157
 Cohen, P. P., 191, 221, 292
 Coleman, J. R., 88
 Comb, D. G., 263, 266, 271, 277, 278, 283, 286, 292, 325, 326, 327, 328, 329, 331, 332, 333, 334, 335, 345, 432, 444, 560
 Cooper, K. W., 450, 453, 464, 470, 565, 572
 Cope, M., 311, 312, 317
 Cowden, R. R., 302, 306, 326, 333, 335
 Cowdry, E. V., 172, 175
 Cox, D. C., 173, 175
 Cox, E. C., 237, 249
 Cronkite, E. P., 378
 Crosby, A. R., 149, 155
 Daleq, A., 313, 317
 Dangeard, P., 68, 71
 Danon, M., 57, 59
 Darnell, J., 20, 27, 153, 156, 201, 207, 227, 228, 233, 384, 385, 455, 470, 482, 483, 485, 486, 492, 493, 494, 498, 503, 504, 506, 507, 513, 515, 523, 529, 530, 531, 533, 543, 554
 Das, C. C., 302, 306
 Das, N. K., 146, 155, 182, 184, 185, 186, 187, 306, 337, 341, 343, 344, 346, 349, 350, 352
 Dauwalder, M., 302, 306
 David, H., 39, 45, 380, 385
 Davidson, E. H., 298, 305, 312, 317, 342, 345, 475, 486
 Dawid, I. B., 229, 233, 298, 299, 300, 305
 Deane, H. W., 415, 418
 Decroly, M., 311, 312, 317, 325, 326, 334
 Dendy, P., 169, 171, 175
 Denny, P. C., 311, 312, 317
 Denues, A. R. T., 127, 133
 Dermen, H., 128, 134
 DeRobertis, E., 415, 418, 422
 Desjardins, R., 109, 117, 193, 194, 195, 207, 210, 246, 247, 250
 Dessev, G. N., 518, 524
 Deuchar, E. M., 425, 428
 DeVallet, J., 333, 335
 Deyrup, I. J., 416, 418
 Dinka, S., 173, 175

- Dodd, M. C., 237, 249
 Doly, J., 292
 Donato, A., 15, 25
 Doty, P., 202, 203, 207, 441, 444
 Dounce, A. L., 117, 246, 247, 249, 250
 Dourmashkin, R., 21, 27
 Doutreligne, J., 69, 71
 Drets, M. E., XVI, 295
 Dubnau, D., 183, 186, 442, 444
 Durwald, H., 120, 27, 182, 186
 Dutta, C. R., 95, 99
- E**
 Eagle, H., 490, 506
 Earle, W. R., 490, 506
 Ecker, R. E., 376, 378
 Edström, J. E., 57, 59, 147, 154, 156, 166, 191, 223, 224, 225, 229, 231, 232, 233, 246, 191, 223, 224, 225, 229, 231, 232, 233, 246, 344, 443, 450, 469, 486, 487
 Eichner, D., 224, 231
 Eicke, J., 169, 174
 Eldjarn, L., 378
 Ellem, K. A. O., 171, 173, 175
 Elsdale, T. R., 301, 306, 390, 398, 399, 411, 417, 432, 443
 Elson, D., 333, 335
 Erikson, R. L., 433, 444
 Errera, M., 20, 27, 182, 186
 Esper, H., 301, 306, 413, 414, 415, 417, 418, 422, 423, 427, 429, 432, 443, 446
 Estable, C., XV, 14, 25, 91, 95, 98, 99, 105, 107, 117, 180, 574
 Evans, H. J., 353, 361
- F**
 Fabbri, F., 68, 70, 126, 133
 Faber, J., 299, 306, 413, 418, 426, 429
 Falk, H., 129, 134
 Farr, A. L., 217, 219
 Favelukes, G., 295
 Favard, P., 184, 187
 Fawcett, D. W., 126, 133
 Feinendegen, L., 190, 210, 283, 323, 366, 367, 378, 430, 512, 544, 560
 Ferguson-Smith, M. A., 414, 418
 Fernandes, M. V., 21, 27, 173, 174, 175
 Ficq, A., 147, 155, 243, 249, 298, 305, 307, 311, 312, 313, 316, 317, 341, 344, 345, 475, 484, 486
 Finamore, F. J., 246, 247, 249, 286, 292
 Fischberg, M., 301, 306, 391, 398, 399, 411, 417, 429, 431, 432, 443
 Fischer, S., 228, 232
 Flaks, J. G., 237, 249
 Flamm, W. G., 217, 219, 246, 247, 249, 251
 Flax, M. H., 149, 156, 341, 344
- Fleissner, E., 226, 232, 271, 277, 278, 286, 292, 427, 429, 450, 470
 Fontana, XV, 1, 9
 Foulds, L., 173, 175
 Fox, C. F., 258, 266
 Fox, F. A., 95, 99
 Fraenkel-Conrat, H., 221
 Franklin, R. M., 21, 27
 Frayssinet, G., 22, 28
 Freed, L., 561
 Frenster, J. H., 19, 26, 216, 219, 224, 232
 Fukada, T., 518, 523
- G**
 Gabrusewycz-Garcia, N., 416, 418
 Galibert, F., 277, 278
 Gall, J. G., 54, 57, 59, 112, 118, 152, 156, 238, 249, 298, 305, 473, 475, 476, 479, 481, 483, 484, 486, 487, 488, 547, 549, 553, 559, 574
 Ganesan, A. T., 433, 444
 Gates, R. R., 67, 69, 70, 184, 187
 Gaulden, M. E., XVI
 Gavaudan, P., 68, 71
 Gay, H., 302, 306
 Georgiev, G. P., 286, 291, 513, 514, 515, 518, 520, 522, 523
 Gershenson, S. M., 450, 470
 Geschwind, I., 359, 361
 Geuskens, M., 259, 266
 Geyer, V. B., 237, 249
 Giacomoni, D., 271, 278, 455, 470
 Gilbert, W., 493, 506
 Gillespie, D., 450, 470
 Girard, M., 228, 233, 384, 385, 482, 483, 485, 486, 498, 503, 504, 507, 529, 530, 531, 533, 543, 554
 Giudice, G., 325, 334
 Glisin, M. V., 325, 326, 334
 Glisin, V. R., 325, 326, 334
 Godman, G. C., 359, 361
 Godward, M. B. E., 116, 118, 129, 134
 Goldstein, L., 147, 155
 Goldstein, M. N., 22, 27
 Gonzalez-Ramirez, J., 15, 18, 25, 26, 111, 115, 117
 Goodman, H. M., 271, 278, 396, 399, 456, 470
 Graham, A., 309, 494, 503, 507, 543
 Grampp, W., 224, 231, 246, 247, 249, 282, 297, 298, 305, 311, 316, 341, 344, 450, 469
 Granboulan, N., 16, 19, 21, 26, 27, 108, 111, 114, 117, 127, 128, 132, 134, 135, 172, 175, 183, 184, 186
 Granboulan, P., 16, 19, 26, 27, 108, 111, 114, 117, 127, 128, 132, 134, 135, 183, 184, 186

- Green, M. M., 463, 471
 Greenberg, H., 238, 249, 489
 Greenman, D. L., 228, 233
 Grisham, J. W., 370, 377
 Grogan, D., 193, 194, 195, 207, 210, 246, 247, 250
 Gropp, A., 14, 15, 25, 127, 133
 Gros, F., 166
 Gross, P. R., 312, 315, 317, 325, 334, 335
 Grunberg-Manago, M., 166
 Gurdon, J. B., 147, 151, 152, 155, 156, 184, 187, 226, 228, 232, 300, 301, 302, 303, 306, 307, 308, 309, 311, 316, 327, 335, 396, 399, 411, 415, 416, 417, 425, 426, 429, 432, 442, 443, 450, 459, 469, 475, 484, 485
 Gustafson, T., 325, 333, 335
 Guttes, E., 184, 187
 Guttes, S., 184, 187
 Guyenot, E., 57, 59

Hadjilov 534, 543
 Hadorn, E., 408, 414, 418
 Haguenauf, F., 14, 15, 21, 25, 27, 91, 99, 127, 133
 Haldane, J. B., 5
 Hall, B. D., 202, 203, 207, 444, 450, 470
 Hammarsten, E., 5
 Handmaker, S. D., 414, 418
 Harkness, R. D., 370, 377
 Harris, H., 19, 26, 109, 117, 228, 232
 Harris, J. I., 221
 Hartman, P. E., 334, 335
 Haruna, I., 209
 Hasselberg, I., 325, 335
 Hay, E. D., 19, 26, 108, 117, 128, 134, 156
 Hayashi, M., 455, 470
 Hayashi, Y., 475, 486
 Hayflick, L., 173, 175
 Hearst, J. E., 441, 444
 Hecht, L. I., 182, 186, 277, 278
 Heidelberger, C., 382, 385
 Heitz, E., 3, 4, 68, 71, 130, 135, 146, 147, 155, 184, 187, 227, 232, 432, 443
 Hell, A., 20, 27, 182, 186
 Henderson, S. A., 342, 345
 Henshaw, E. C., 154, 157, 522, 524, 535, 544
 Herbert, E., 277, 278
 Herick, R., 94, 99
 Hess, O., 150, 152, 156
 Hiatt, H. H., 154, 157, 201, 207, 513, 522, 523, 524, 535, 544
 Higashi, K., 193, 207, 247, 250, 256
 Higgins, G. M., 370, 377
 Higginson, J., 21, 27, 380, 385
 Himes, M. H., 149, 156, 341, 344
 Hinz, R. N., 21, 27
 Hirschman, R., 380, 385
 Hnilica, L. S., 224, 231, 360, 361
 Hodnett, L., 194, 202, 207
 Hoecker, G., 11, 33
 Hollaender, A., XI, XVI, 573
 Holland, I. B., 209
 Holley, R. W., 336
 Holtzman, E., 238, 249, 489, 507, 508
 Hopkins, J. W., 153, 157, 166, 227, 228, 232, 233, 337, 342, 344, 375, 378, 432, 435, 444, 450, 460, 470, 530, 535, 541, 543
 Hruban, Z., 22, 28
 Hsu, T. C., 97, 99, 105, 312, 317
 Huang, P. C., 354, 361, 454, 460, 470, 518, 519, 524
 Hubbard, M., 312, 315, 317
 Hughes, A., 110, 117
 Hughes, B., 22, 27
 Hughes, W. L., 378
 Hull, B. D., 433, 434, 444
 Hultin, T., 311, 317, 325, 334
 Humphrey, G. B., 286, 287, 292
 Humphrey, R. M., 286, 287, 292, 312, 317
 Humphrey, R. R., 416, 418
 Hungerford, D. A., 168, 175
 Hurlbert, R. B., 224, 231, 360, 361
 Hyde, B. B., 39, 40, 41, 42, 45, 52, 68, 70, 126, 133, 183, 186, 210, 214, 215, 219, 226, 232, 246, 247, 249, 337, 342, 344, 432, 444, 507, 508, 520, 524, 530, 543, 550, 554
 Hyden, H., 147, 149, 155

Infante, A., 535, 542, 544, 570, 572
 Iñiquez, P. A., 95, 99
 Ito, S., 417, 419
 Izard, J., 15, 25
 Izawa, M., 57, 59, 112, 118, 298, 305, 479, 481, 483, 484, 486
 Izquierdo, L., 18, 26

Jacob, J., 20, 27, 39, 45, 69, 71, 127, 128, 134, 184, 186, 187, 226, 228, 232, 246, 249, 255, 256, 257, 258, 261, 263, 264, 265, 266, 267, 269, 271, 277, 278, 327, 335, 396, 399, 427, 429, 431, 450, 457, 470, 528, 543, 567, 572
 Jacob, M., 390
 Jacob, S. T., 193, 246, 247, 250
 Jezequel, A. M., 22, 28
 Johnson, 561

- Joklik, W. K., 154, 157, 531, 543
 Jones, K. W., 151, 156, 184, 186, 226, 232, 256, 263, 265, 390, 432, 443
 Jordan, E. G., 129, 134
 Journey, L. J., 22, 27
 Judd, B. H., 463, 471
 Julien, J., 299, 305
 Justus, J. T., 416, 418

K
 Kabat, S., 454, 460, 470, 518, 519, 524
 Kahn, J., 412, 417, 432, 442, 443, 446
 Kaji, A., 553
 Kaji, H., 553
 Kalnins, V. I., 127, 128, 132, 134, 224, 231
 Kano-Sueoka, T., 334, 335
 Kaplan, W. D., 414, 418
 Karasaki, S., 20, 27, 127, 128, 132, 134, 142, 151, 156, 183, 184, 186, 302, 306
 Karney, D., 22, 28
 Kasten, F. H., 142, 180, 190, 353, 354, 355, 359, 360, 361, 367, 368, 423, 424
 Kato, K.-I., 226, 232, 256, 261, 263, 265, 266
 Katz, S., 263, 266, 271, 277, 278, 286, 292, 325, 326, 327, 329, 331, 332, 333, 334, 335, 432, 444
 Kaufmann, B. P., 4, 302, 306
 Kawade, Y., 518, 523
 Kay, E. R. M., 109, 117, 247, 250
 Kelley, D. E., 232, 237, 395, 399, 429, 528, 535, 536, 543
 Kenney, F. T., 228, 233
 Kent, R., 477, 486
 Kezer, J., 484, 486
 Kikkava, H., 464, 471
 Kiknadze, I. I., 302, 306
 Kinoshita, R., 414, 418
 Kiriyama, M., 13, 25, 127, 134
 Kitazume, Y., 548
 Kleinfeld, R. G., 147, 155, 156, 182, 186, 369, 370, 373, 375, 376, 377, 378
 Klevecz, R. R., 97, 99, 105
 Kopac, M. J., 416, 418
 Koulis, S., 369, 370, 371, 373, 375, 376, 377
 Krivshenko, J., 463, 471
 Kroeger, H., 256, 266, 416, 419
 Kruse, P. F., 168, 174
 Krychevskaya, A. A., 521, 522, 524
 Kurland, C. G., 248, 250
 Küster, E., 128, 134

L
 Lafarge, C., 22, 28
 Lafontaine, J. G., 11, 39, 43, 44, 45, 67, 68, 69, 70, 71, 96, 99, 126, 129, 131, 133, 142, 143, 146, 155, 184, 187
 Laird, A. K., 369, 376, 377
 Lapis, K., 22, 28
 Lara, F. J., 11, 559
 Larsen, C. J., 277, 278
 Larsen, K., 153, 156
 Lasansky, A., 415, 418, 422
 Latham, H., 19, 27, 201, 207, 227, 384, 385, 482, 485, 486, 493, 494, 503, 504, 506, 507, 513, 515, 523, 529, 530, 531, 533, 543
 La Torre, J. L., 182, 186
 Laughnan, J. R., 465, 471
 Leboy, P. S., 237, 249
 Leblond, C. P., 29, 27, 152, 153, 156, 371, 377
 Le Breton, E., 22, 28
 Ledbetter, M. C., 148, 156
 Lederberg, J., 433, 444
 Ledig, M., 190
 Leduc, E. H., 16, 25, 27, 128, 134
 Lehman, H. E., 302, 306, 326, 333, 335
 Lelong, J. C., 277, 278
 Lerman, M. I., 286, 291, 513, 514, 518, 523, 535, 543
 Lettré, R., 37, 91, 99, 105, 107, 108, 117, 367, 390, 422
 Levinthal, J. D., 21, 27
 Levy, A. L., 221
 Lewis, E. B., 456, 463, 470
 Lewis, H. W., 473, 547, 557, 561
 Lewis, U. F., 218, 219, 237, 249
 Lewis, W. H., 422
 Liau, M. C., 193, 195, 206, 224, 231, 360, 361
 Lieberman, I., 247, 250, 286, 291, 369, 375, 377
 Lima-de-Faria, A., 461, 471
 Lin, M., 9, 147, 152, 155
 Lindenmeyer, G., 194, 204, 207, 247, 250, 286, 292
 Lindsley, D. L., 59, 399, 449, 543
 Lippe, B., 382, 385
 Litt, M., 117, 246, 247, 249, 250
 Littau, V. C., 19, 26
 Littna, E., 298, 299, 300, 301, 305, 306, 312, 317, 425, 426, 429, 436, 441, 444, 445, 475, 476, 477, 478, 479, 485, 486
 Lloyd, L., 54, 59, 298, 305, 476, 486
 Loewenstein, W. R., 417, 419
 Longwell, A. C., 9, 147, 155, 184, 187
 Lord, F. A., 67, 68, 69, 70
 Love, R., 15, 21, 25, 27, 105, 167, 168, 171, 173, 174, 175, 180, 182, 185, 190, 367, 368
 Løvlie, A., 235
 Lowry, O. H., 217, 219
 Lowy, B. A., 228, 232

- Luft, J. H., 126, 133
 Luykx, P., 337, 344
MacGregor, H. C., 54, 59
 McCarthy, B. J., 434, 444
 McClendon, D., 22, 28
 McClintock, B., 4, 9, 59, 69, 71, 130, 135,
 146, 147, 148, 149, 151, 152, 155, 156, 184,
 187, 227, 232, 391, 423, 432, 443, 446, 463,
 471
 McConkey, E. H., 157, 166, 227, 228, 232,
 233, 337, 342, 344, 375, 378, 432, 434, 444,
 450, 470, 530, 535, 541, 543
 McCoy, T. A., 168, 174
 McElhinney, A. J., 182, 186
 McLeish, J., 68, 71, 413, 418
 McMaster-Kaye, R., 147, 154, 155, 182, 186
 Maggio, R., 129, 134, 193, 207, 213, 214, 216,
 219, 221, 246, 247, 249, 250, 312, 315, 317
 Malkin, L. I., 312, 315, 317
 Mancuso, V., 302, 306
 Mandel, P., 36, 166, 190, 209, 221, 292, 308,
 390, 406, 445, 487, 488, 559, 560
 Mangan, J., 325, 334
 Mantieva, V. L., 286, 291, 513, 514, 515, 523
 Manton, I., 69, 71
 Margaud, L., 488
 Marinozzi, V., 15, 16, 17, 20, 25, 26, 39, 45,
 128, 134, 183, 186
 Markert, C. L., 302, 306
 Markow, G. G., 518, 524
 Marmur, J., 183, 186, 433, 441, 442, 444
 Marquisee, M., 336
 Marshak, A. G., 69, 71
 Martin, R. G., 479, 486
 Mateyko, Gladys M., 416, 418
 Maxwell, M., 168, 174
 Mazanec, K., 18, 26
 Mead, C. G., 229, 233
 Meehelke, F., 394, 396, 398, 400
 Meiners, M. L., 169, 174
 Meischer, F., 2
 Meisel, E., 94, 99
 Mellman, W. J., 168, 175
 Meselson, M., 441, 444
 Metz, C. W., 394, 398
 Meyer, G. F., 81, 88, 150, 152, 156
 Micou, J., 147, 155
 Miki-Noumura, T., 325, 334
 Miller, O. L., XI, XIII, XVI, 53, 56, 57,
 59, 66, 184, 187, 224, 231, 461, 471, 484,
 486
 Mills, D., 209
 Mintz, B., 302, 307, 308, 416, 418
 Mirsky, A. E., 19, 26, 57, 59, 112, 118, 216,
 219, 224, 232, 258, 266, 298, 305, 312, 317,
 342, 345, 475, 479, 481, 483, 484, 486
 Monier, R., 299, 305
 Monroy, A., 311, 312, 315, 316, 317, 325, 334
 Montagnier, L., 518, 524
 Montgomery, P. O. B., 22, 27, 28
 Montgomery, T. H., 1, 2, 4
 Monty, K. J., 109, 117, 246, 247, 249, 250
 Moorhead, P. S., 168, 173, 175
 Morasca, L., 353, 361
 Moses, M. J., 88
 Mottram, F. C., 127, 133
 Moulé, Y., 415, 418
 Mundell, R., 235
 Muramatsu, M., 193, 194, 195, 196, 199,
 202, 203, 206, 207, 246, 247, 250, 286, 287,
 291, 292
 Murray, K., 327, 335
Nadler, N. J., 153, 156, 377
 Nakada, D., 551
 Nakamoto, T., 258, 266
 Nakken, K. F., 378
 Nancy, M. T., 18, 26
 Nash, D., 269
 Navaschin, M., 4, 566, 572
 Nemer, M., 326, 335, 535, 542, 544, 570, 572
 Newton, A. A., 168, 175
 Nieuwkoop, P. D., 299, 306, 413, 418, 426,
 429
 Noll, H., 442, 444
 Nomura, M., 248, 250
 Novelli, G. D., 553
 Nowell, P. C., 168, 175
 Nygaard, A. P., 378, 433, 434, 444
Oberling, C., 14, 15, 25, 91, 94, 99, 127,
 133
 Ohno, S., 414, 418
 Oishi, M., 442, 444, 551
 Okamura, N., 206, 207, 369, 375, 376, 377,
 395, 399
 Osawa, S., 258, 266, 475, 486
 Oura, H., 441, 444
 Ozban, N., 312, 317, 323
Painter, T. S., 56, 57, 59, 66
 Palade, G. E., 129, 134, 193, 207, 214, 216,
 219, 246, 247, 249, 250
 Paletta, F. X., 172, 175
 Panijel, J., 237, 248, 249
 Panitz, R., 396, 399

- Pankova, N. V., 302, 307
 Parthasarathy, 423
 Pavan, C., 36, 52, 66, 105, 268, 350, 391,
 408, 422, 447, 487
 Paweletz, N., 107
 Peacock, W. J., 57, 58, 59, 461, 471
 Pelling, C., 147, 153, 155, 156, 184, 187, 210,
 227, 232, 267, 268, 269, 283, 393, 394, 395,
 398, 400, 402, 408, 409, 488
 Penman, S., 210, 228, 233, 238, 249, 252,
 384, 385, 429, 430, 473, 482, 483, 485, 486,
 488, 489, 498, 499, 503, 507, 508, 512, 529,
 530, 531, 533, 543, 547, 548, 550, 552,
 553, 554, 556, 558, 559, 560
 Perry, R. P., XVI, 20, 26, 27, 36, 37, 157,
 166, 180, 182, 186, 201, 207, 208, 221, 222,
 226, 227, 228, 232, 238, 249, 251, 255, 265,
 266, 267, 284, 293, 308, 309, 311, 316, 327,
 335, 375, 376, 378, 395, 398, 399, 429, 437,
 441, 443, 445, 450, 459, 469, 473, 482, 484,
 485, 486, 513, 514, 515, 523, 527, 528, 529,
 530, 533, 534, 535, 536, 539, 542, 543,
 544, 545, 547, 548, 549, 550, 551, 552, 553,
 554, 555, 556, 557, 558, 559, 560, 561, 574
 Petermann, M. L., 235, 248, 549
 Peters, K., 127, 134
 Peterson, H. M., 465, 471
 Pinzino, C. J., 325, 326, 327, 329, 331, 333,
 335
 Plaut, W., 269
 Porter, K. R., 13, 24, 67, 68, 70, 126, 133,
 148, 156, 415, 418
 Porter, R. P., 302, 307
 Potter, V. R., 182, 186
 Poulson, D. F., 384, 398, 403
 Poussel, H., 68, 71
 Prensky, W., 359, 361
 Pulitzer, J. F., 153, 157

Quertier, J., 312, 317, 325, 334
 Quirin, C., 390

Rabotti, G., 171, 175
 Rabson, A. S., 173, 175
 Rake, A., 494, 503, 507, 543
 Ramuz, M., 292
 Randall, R. J., 217, 218, 219
 Ranzi, S., 326, 335
 Rasch, E., 147, 153, 155, 156, 341, 343, 344
 Rather, L. J., 369, 377, 378
 Rattenbury, J. A., 69, 71, 129, 135, 146, 155
 Raven, C. P., 298, 301, 302, 305, 306
 Rebhun, L., 147, 155, 341, 343, 344
 Reich, E., 376, 378
 Reisfeld, R. A., 218, 219, 237, 249
 Rekemeyer, M. L., 407
 Resende, F., 116, 118
 Revel, J. P., 19, 26, 108, 117, 127, 134
 Revel, M., 154, 157, 522, 524, 535, 544
 Reynolds, R. C., 22, 27, 28
 Rho, J. H., 227, 232, 550
 Rich, A., 271, 278, 396, 399, 456, 470
 Richards, A., 302, 307
 Rimbaut, C., 22, 27
 Rinaldi, A. M., 312, 315, 317
 Ris, H., 298, 305
 Ritossa, F. M., 8, 57, 59, 109, 117, 147, 152,
 153, 155, 157, 166, 183, 186, 224, 227, 231,
 309, 311, 316, 327, 335, 337, 342, 344, 396,
 397, 399, 407, 432, 441, 442, 444, 445, 446,
 447, 449, 450, 452, 454, 455, 456, 459, 461,
 469, 470, 471, 472, 515, 523, 528, 543
 Ro, T. S., 193, 199, 204, 207, 246, 247, 250,
 286, 291, 292, 293
 Robineaux, R., 22, 27
 Rodkiewicz, B., 126, 133
 Rolfe, R., 441, 444
 Rosebrough, N. J., 217, 219
 Rosset, R., 290, 305
 Roth, J. S., 290, 292
 Rowell, N. R., 186
 Rudkin, G. T., 152, 156, 454, 470
 Rusch, H. P., 184, 187
 Ruthmann, A., 341, 344
 Ryberg, H., 383, 385

Saez, F. A., XVI, 52, 352
 Saksela, E., 173, 175
 Salomon, J. C., 17, 23, 27, 28
 Samarina, O. P., 513, 514, 520, 422, 523,
 524
 Sankaranaryanan, K., 21, 27, 39, 41, 43,
 45, 68, 70, 126, 133, 183, 186, 507, 507
 Sarkar, N., 333, 335
 Sawoda, T., 13, 25, 126, 134
 Scherrer, K., 20, 27, 153, 156, 207, 455, 470,
 482, 486, 488, 492, 493, 494, 498, 503, 506,
 513, 515, 523
 Schin, K. S., 77, 81
 Schoeff, G., 22, 27
 Schor, N., 224, 231, 246, 247, 249, 282, 298,
 305, 311, 316, 341, 344, 450, 469
 Schrandt, 417, 419
 Schreiber, G., 191, 309
 Schultz, J., 1, 11, 88, 89, 252, 253, 407, 475,
 485
 Schumacher, B. L., 302, 307
 Serra, J. A., 69, 71, 126, 129, 133, 135, 146,
 155
 Severtzov, A. N., 513, 514, 523

- Sexton, W. A., 378
 Shafiq, A., 376, 378
 Shein, H. M., 21, 27
 Shortman, K., 291, 292
 Siebert, G., 194, 195, 196, 197, 198, 199,
 200, 201, 202, 203, 204, 207, 247, 259, 285,
 286, 287, 288, 292, 293, 512, 560
 Siebs, W., 91, 99, 107, 108, 117, 422
 Siegel, E. P., 337, 344
 Siegesmund, K. A., 95, 99
 Siekevitz, P., 129, 134, 183, 207, 213, 214,
 216, 219, 246, 247, 249, 250
 Silver, D. J., 325, 334
 Simard, R., 22, 28
 Sirlin, J. L., 20, 27, 36, 39, 45, 57, 59, 66,
 69, 71, 127, 128, 134, 142, 180, 182, 183,
 184, 186, 187, 190, 209, 226, 228, 232, 235,
 246, 248, 249, 255, 256, 257, 258, 261, 263,
 264, 265, 266, 267, 268, 269, 271, 277, 278,
 282, 307, 312, 317, 323, 327, 335, 336, 351,
 396, 399, 426, 427, 429, 431, 450, 457, 470,
 472, 528, 543, 557, 566, 572, 574
 Siskin, J. E., 353, 361
 Slesers, A., 22, 28
 Smetana, K., 13, 15, 16, 25, 26, 58, 59, 109,
 117, 193, 194, 195, 206, 207, 286, 287, 292,
 337, 344
 Smirnov, M. N., 513, 514, 523
 Smith, C. L., 168, 175
 Smith, I. H., 183, 186, 238, 249, 442, 444,
 489, 507, 508
 Smith, H. H., 359, 361
 Smith, S., 301, 306, 398, 399, 411, 417, 432,
 443
 Somers, C. E., 312, 317
 Sotelo, J. R., 14, 25, 77, 78, 81, 88, 91, 98,
 117
 Soudek, D., 128, 134
 Spencer, W. P., 466, 471
 Spiegelman, S., 57, 59, 109, 117, 147, 155,
 183, 186, 209, 224, 227, 231, 271, 278, 311,
 316, 326, 335, 337, 342, 344, 396, 399, 432,
 434, 441, 442, 444, 450, 452, 454, 455, 456,
 459, 461, 469, 470, 471, 515, 523, 543
 Spirin, A. S., 522, 524, 533, 534, 542, 543,
 544, 553, 570, 572
 Srinivasan, P. R., 227, 232, 395, 399, 429,
 528, 543
 Staehelin, T. H., 441, 444
 Steele, W. J., 193, 194, 198, 204, 206, 207,
 210, 246, 247, 250, 286, 291, 292, 293, 369,
 375, 376, 377, 378, 395, 399, 524
 Steffensen, D., 463, 471
 Stenram, I., 383, 385
 Stenram, U., 379, 380, 381, 383, 385, 386,
 390, 418
 Stent, G., 333, 335
 Stephens, S. G., 456, 463, 470
 Stephenson, M. L., 277, 278
 Stern, C., 464, 471
 Stern, H., 375, 378
 Stevens, B. J., 17, 26, 44, 45, 69, 71, 131,
 132, 135, 145, 152, 153, 156, 157, 184, 187,
 224, 231, 558
 Stich, H. F., 126, 127, 129, 134, 231
 Stinebring, W. R., 237, 249
 Stöcker, E., 16, 25, 128, 134, 380, 385
 Strassburger, E., 2
 Strasser, F. F., 190, 353, 354, 359, 361
 Studzinski, G. P., 168, 171, 173, 174, 175,
 185, 190
 Sturtevant, A. H., 463, 471
 Sueoka, N., 334, 335, 441, 442, 444, 551
 Sugihara, R., 15, 25, 69, 71, 127, 134
 Sugioka, M., 15, 25, 69, 71, 134
 Suskind, R. G., 111, 118, 367, 551
 Suter, E., 23, 28
 Svedberg, 5
 Svihla, G., 9, 147, 155, 184, 187
 Svoboda, D., 21, 27, 380, 385
 Swift, H., 16, 20, 22, 25, 28, 37, 45, 69, 71,
 127, 128, 132, 134, 135, 142, 145, 152, 153,
 155, 156, 157, 166, 183, 186, 209, 235, 248,
 267, 341, 343, 344, 350, 368, 414, 418, 423,
 454, 470, 507, 508, 558, 573
 Szego, C., 382, 385
 Szollosi, D., 18, 26
 Szybalski, W., 433, 444
Tandler, C. J., 181, 182, 184, 185, 186, 190,
 226, 232, 246, 249, 255, 256, 257, 261, 263,
 264, 265, 266, 271, 277, 278, 312, 317, 323,
 350, 470
 Taylor, A. N., 57, 59, 66
 Taylor, J. H., 11, 52, 336, 353, 361, 371,
 377, 390, 545, 557
 Tencer, R., 311, 312, 316, 317
 Terskich, V. V., 518, 523
 Thoenes, W., 16, 25, 128, 134, 380, 385
 Totter, J. R., 578
 Tournier, P., 16, 21, 26, 27
 Trakatellis, A. C., 237, 249
 Travaglini, E., 9
 Trosko, J. E., 463, 471
 Trujillo, J. M., 414, 418
 Trujillo-Cenoz, O., 78, 81
 Tsanev, R. G., 518, 524
 Tsukada, K., 247, 250, 286, 291, 369, 375,
 377
 Tubergen, R. P., van, 19, 26
 Tuft, P., 416, 418
 Tumanyan, B. D., 513, 519, 523

- Turner, M., 354, 355, 361
 Tyler, A., 311, 312, 317
- V**alencia, J. I., VII, XIII, 295
 Valeva, L. I., 518, 524
 Vannfält, J., 380, 385
 Venkov, P. V., 518, 524
 Vermeulen, C. W., 459, 470
 Vial, J. D., 18, 26
 Villalobos, J. G., 193, 204, 207, 247, 250, 285, 286, 291, 292, 293, 376, 378
 Vincent, W. S., XI, XIII, XVI, 7, 112, 118, 153, 156, 166, 221, 222, 225, 226, 227, 232, 235, 236, 243, 246, 247, 248, 249, 250, 251, 252, 253, 285, 286, 291, 292, 341, 344, 416, 418, 432, 444, 445, 447, 473, 487, 544, 547, 548, 550, 552, 554, 555, 559, 560, 578
 Vinograd, J., 441, 444, 477, 486
 Vittorelli, M. L., 325, 334
 Vladimirtseva, E. A., 523
 Volkin, E., 229, 233
 Von Borstel, R. C., XIII, XVI, 153, 157, 209, 250, 259, 295, 409
 Von Haam, E., 147, 155, 186, 370, 371, 377
- W**achstein, M., 94, 99
 Waddington, C. H., XV, 283, 307, 308, 309, 390, 415, 418, 447, 471, 563
 Wallace, H., 59, 184, 187, 227, 232, 301, 302, 306, 307, 308, 397, 399, 412, 414, 416, 417, 418, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 436, 441, 443, 444, 460, 463, 471
 Waller, J. P., 244, 249, 327, 335
 Walsh, R. J., 15, 25, 167, 168, 171, 174
 Warner, J., 552
 Watson, J. D., 248, 250
 Watson, M. L., 16, 21, 25
 Watts, J. W., 228, 232
- Weigle, J., 477, 486
 Weiss, S. B., 258, 266
 Weissenfels, N., 18, 26, 108, 117
 Wessel, G., 325, 334
 Wettstein, F. O., 441, 444
 Wettstein, R., 77, 78, 81
 White, J. L., 460, 471
 Wicker, R., 21, 27
 Wicks, W. D., 228, 233
 Weisner, G., 376, 378
 Wildy, P., 173, 175, 367
 Willén, R., 381, 385
 Williams, D. E., 218, 219, 237, 249
 Willis, R. A., 171, 175
 Willmer, E. N., 416, 419
 Wilson, E. B., 2
 Wilt, F. H., 326, 335
 Winiwarter, H., de 78, 81
 Wolff, S., 463, 471
 Woodard, J. W., 153, 155, 156, 344
 Woods, P. S., 153, 156, 182, 186, 271, 272, 273, 274, 278, 371, 377, 528, 543
 Work, T. S., 244, 249
 Wrba, H., 171, 174
 Wrinch, D., 5
- Y**ankofsky, S. A., 434, 441, 442, 444, 450, 454, 456, 459, 460, 463, 470, 471
 Yasuzumi, G., 3, 25, 69, 71, 126, 127, 134
 Yčas, M., 548
 Yoshikawa, M., 518, 523
- Z**acharias, E., 2, 3, 7
 Zalokar, M., 312, 317
 Zamecnik, P. C., 277, 278
 Zamur, A., 336
 Zirkle, C., 67, 68, 70
 Zubay, G., 271, 272, 273, 274, 278, 528, 543

SUBJECT INDEX

- A**ccessory body, of Cajal, 96
 Accessory nucleolus, 396
 function in anucleolate genome, 396
 in *Urechis* oocytes, 341, 342
 Accessory nucleolus organizer, 397, 406
Acetabularia
 nucleotide composition,
 of cytoplasm, 226
 of DNA, 226
 of nucleoli, 226
 Acid phosphatase, 285, 287
Acricotopus lucidus, salivary gland chromosomes of, 394, 401
 ACTH, actinomycin effect on secretion of, 382
 Actidione
 effect on
 protein synthesis, 552
 uridine incorporation, 552
 Actinomycin
 effect on
 amino acid feeding, 380
 appetite, 380
 Balbiani ring RNA synthesis, 396
 cytidine incorporation, 532
 HeLa cell RNA, 498
 hybridization, 429
 liver cell RNA, 382, 515, 559, 560
 mouse embryo, 308
 nucleolar morphology, 22, 23, 170, 171, 380
 amphibian embryonic cells, 390
 liver cells,
 Walker tumor cells
 nucleolar RNA synthesis, 227, 228, 261, 262, 298, 515
 Øestradiol-induced protein synthesis, 382
 protein synthesis, 381, 553
 ribonuclease, 289, 290
 RNA polymerase, 390
 RNA precursor incorporation, 266, 267
 RNA synthesis, 552, 553, 556, 558, 559, 560
 rRNA migration, 532, 533
 RNP granules, 209
 40S RNP, 539
 uridine incorporation, sea urchin oogenesis, 314, 320, 498, 504, 505
 nucleolar autoradiograph, 210
 Adenine, effect on terminal incorporation, 226
 Adenosine
 effect on
 cytoplasmic ribosomes, 383, 384
 endoplasmic reticulum, 383
 metabolism, 390
 mitochondria, 383
 nucleolar morphology, 380, 383
 nucleolonema, 383
 pars amorpha, 383
 perichromatin granules, 383
 polysomes, 383, 384
 in tissue culture cells, 383, 384
 Adenosine deaminase, 285
 Adenylate kinase, 287
 Aflatoxin, effect on nucleolar morphology, 22
 Alanine, incorporation into sea urchin basic protein, 328
 Alanyl-glycine dipeptidase, 285
 Alkali-labile incorporation
 of leucine, 226
 of lysine, 226
 Alkaline phosphatase, 285, 414
Allium cepa
 Feulgen staining in, 126
 metachromatic staining, 126
 methylene blue staining, 126
 nucleolar architecture, 125
 nucleolar fibrils, 125
 nucleolonema, 125
 RNA synthesis in, 338, 339
 root meristem nucleoli, 125
 vacuoles in pars amorpha, 132
Amblystoma mexicanum, carrier RNA, 476
 Amino acid activating enzymes, 286

- Amino acids
 content of
 histones, 195, 205
 liver nuclei, 195, 205
 liver nucleoli, 195, 205
 Walker nuclei, 195, 205
 Walker nucleoli, 195, 205
 nucleolar proteins, 205
 effect on starved liver cell morphology, 380
 incorporation
 in cell cycle, 353
 in isolated nucleoli, 221
 of *Asterias*, 221
 of guinea pig liver, 214
 puromycin effect on
 guinea pig nucleoli, 214
 pea nucleoli, 214
- Aminopterin, effect on nucleolar morphology, 22
- AMP incorporation, into RNA, 258
- Amphibian oocyte
 nucleolar DNA in, 224
 nucleolar extrachromosomal DNA in, 57
 number of nucleoli in, 298
- Anaphase, RNA on chromosomes, 152
- Aneuploid cells, nucleolini differences in, 172
- Angular nucleoli, in *P. ovata*, 43
- Anisonucleolinosi
 cell strains exhibiting, 169, 170
 nucleolini size in diploid cells, 169, 171
 tissue culture cells exhibiting, 168
- Annulate lamellae, 37
- Antibodies
 to nucleolar proteins, 237
 to ribosomal proteins, 237, 242
- Antigens common to nucleolar and ribosomal proteins, 238
- Antimetabolites, effect on nucleolini, 173
- Anucleolate mutant, *see* Nucleolus Organizer Mutant, Chironomid Embryo Development, 397, 405
- Anucleolate nuclei, 152, 153, 154
- Arbacia*, RNA synthesis during oogenesis, 312
- Argentophilia, 350
 fibers, 350, 14
 nucleolonema, 14
- Arginine
 incorporation in cell cycles, 354, 356
 synthesis inhibition by canavanine, 173
- Armadillidium nasatum*
 nucleolonema, 100, 102
 pars amorphia, 102
- Ascidia*, nucleolar appearance in, 302
- Ascites cells
 P³² incorporation in, 37
 thymidine incorporation in, 37
- Association nucleolus
 meiosis of Gryllidae, 79
- Asterias*
 nucleoli, 235
 DNA content of, 247
 isolation of, 236
 L-Protein in, 236
 nucleotide composition, 226
 protein content of, 247
 RNA, 238
 ribosomal, 238, 239
 synthesis, TRB inhibition of, 259
 transfer RNA binding, 221
- oocyte
 nucleotide composition
 cytoplasmic RNA, 226
 DNA, 226
 ribosomal RNA, 226, 238
 ribosomes, 235
 RNA fractions, 153, 225
 RNA synthesis in, 312, 313
- Adenosinetriphosphatase
 A, 287, 288
 B, 287, 288
 in nucleolonema, 94
 in pars amorphia, 94
- Australorbis glabratus* Say, 309
- Autoradiography
 anucleolate mutant, *Xenopus*, 425
 of culture cells, 353
 cytidine in *Vicia faba*, 274
 DNA synthesis in *Smittia*, 256
 nucleolar, 18
 protein synthesis in *Smittia*, 256
 regenerating liver, 369, 379
 uridine incorporation in *Smittia*, 256, 257, 258
 of virus infected cells, 21
- Azaguanine
 effect on
 nucleolar morphology, 22
 rRNA, 559
 45S RNA breakdown, 529
- Azaserine, effect on nucleolar morphology, 22
- Azure B staining, maize nucleolus, 149, 351, 415

- albani ring
- actinomycin effect on RNA synthesis in, 396
- in *Chironomus*, 342
- formation of, 394, 395
- nucleolini relationship to, 180
- nucleotide composition of, 230
- uridine incorporation in, 395
- Base composition, *see* DNA, RNA Composition
- Bacillus subtilis*, RNA cistron number, 551
- Basophilia, azure B, 41
- Benzimidazoles
 - effect
 - on chromosomal RNA, 226
 - on newly synthesized RNA, 260, 267
- Binucleolate microspores, in maize, 150
- Blastocladiella emersonii*, methyl accepting RNA ribosomes, 333
- Blastoderm, nucleolar appearance in, 302
- Blastula
 - mRNA synthesis in sea urchin, 328, 334
 - nucleolar appearance in, 302
 - rRNA synthesis in sea urchin, 312
- "Blobs", 307, 350, 416, 422, 423, 566
 - RNA in nucleolus organizer mutant, *Xenopus*, 427, 428, 430
- "Bobbied" mutant, *Drosophila*, 407, 471, 472, 565
 - rRNA cistron relationship, 463, 471, 472
- Bradysia*, nucleolar material location in chromosome, 566, 567
- Buoyant density, RNP particle analysis of, 534, 535, 536
- C**anavanine
 - arginine synthesis inhibition of, 173
 - effect on nucleolar morphology, 170, 171
- Cancer cell
 - nucleolar hypertrophy, 21
 - nucleolar pathology, 21
- Carcinogen, effect on nucleolini, 173
- Cat, nucleolonema, 100
- Catalase, 287
- Cell cycle
 - incorporation during
 - of arginine, 356
 - of glycine, 359
 - of leucine, 358
 - of lysine, 357
 - of thymidine, 355
 - of tryptophane, 358
 - nucleolar incorporation
 - of cytidine, 366
 - of uridine, 366
 - ribosome synthesis, time of, 552
 - rRNA synthesis in, 560
- Cesium chloride gradient centrifugation, 434, 527
- Chicken
 - fibroblast
 - nucleoli in, 110
 - nucleolonema, 113
 - nucleolar appearance in, 302
 - rRNA cistron number in, 461
- Chironomus*
 - anucleolar embryo development of, 397, 405
 - Balbani Ring, 342
 - fibrillar RNA component of, 184
 - malpighian tubule chromosomes in, 394, 401
 - nucleolar appearance in, 302
 - nucleolar incorporation in, 267
 - nucleolar RNA synthesis in, 20
 - nucleolus organizer in, 223, 393
 - nucleotide composition of
 - cytoplasm, 226
 - DNA, 226, 230
 - nucleoli, 226, 230
 - polytene chromosomes in, 153
- Chiton*, nucleolar appearance in, 302
- Chloramphenicol, effect on nucleolar morphology, 22
- Chondrocytes
 - nucleolar morphology, 421
 - nucleolar size, 421
- Chortophaga*, nucleolus, 43
- Chromatin
 - fibrils in nucleoli, 128
 - inclusions in nucleoli, 108
 - intranucleolar, 16, 68
 - staining of, 114
 - thymidine incorporation of, 19
 - uridine incorporation of, 19
 - lamellae, 110
 - of nucleoli, 115
 - uridine incorporation in nucleolus organizer mutant, *Xenopus*, 426
- Chromatography
 - alanine-C¹⁴ incorporation into protein, 328, 329
 - calf thymus histones of, 328, 329
 - sea urchin proteins, 328, 329
 - tRNA synthesis of, 257
- Chromomeres, autoradiograph of, 487
- Chromosome
 - deletion of, 564
 - DNA
 - P³² incorporation into, 37
 - thymidine incorporation into, 37

- Chromosome—Continued
 lampbrush, vertebrate oocytes, 152
 loops, 487
 in malpighian tubule, 394
 matrix in nucleolus formation, 146
 "micronucleoli" on, 406
 morphology, factors modifying, 109
 nucleolar attachment, 394
 nucleotide composition of
 Chironomus, 230
 Drosophila, 230
 number, nucleolar morphology relationship, 173
 pachytene, 148, 153
 polytene
 of *Chironomus*, 153
 of *Drosophila*, 153
 puffs
 nonhistone protein proportion, 447
 RNA proportion, 447
 RNA
 in anaphase, 152
 in telophase, 152
 of salivary gland, 394
Ciona, nucleolar appearance in, 302
 Cistrons
 for nucleolar RNA, 224
 ribosomal, 183
 Cleavage
 effect of puromycin on, 312
 nucleolar appearance in, 302
 RNA synthesis in sea urchin, 325, 326
 CMP culture cells, synchrony of, 354
 Colchicine, effect on nucleolar morphology, 170, 172
 Contamination
 of isolated nucleoli, 242
 catalase as marker enzyme, 286
 of nuclei with 16S RNA, 503
 "Convertase", 204, 209
Coregonus, nucleolar appearance in, 302
 Corpuscle, of Levi-Barr, 95
 Cortical zone nucleoli, of *P. ovata*, 43, 49, 51
 Cortisone, effect on liver regeneration, 382
Crepis, organizer competition, 566
 Crossing-over, unequal, effect on rRNA cistrons, 467, 471
Cyclops, nucleolar appearance in, 302
 Cytidine incorporation
 into chromosomal RNA, 147
 into *Drosophila* nucleolus, 147
 L-cell RNP, 530, 531
 in partial hepatectomy, 370
 into 40S RNP, 539
 during sea urchin oogenesis, 314, 321, 322
 into tRNA, 274, 275
 in tissue culture cells, 182
 in *Zea mays*, 349
 Cytology, nucleolus organizer region, *Xenopus*, 411, 412
 Cytoplasmic ribosomes, nucleolar synthesis of, 384
 Cytoplasm, RNA precursor incorporation in sea urchin oocyte, 315
 Cytosine, terminal addition in nucleolar RNA, 226
 Deoxycorticosterone acetate
 effect with cortisone
 on actinomycin inhibition of liver regeneration, 382
 on liver regeneration, 382
 Deoxypyrimidine, 512
 Deoxyribonuclease, 286, 287
 effect on nucleolar morphology, 56
 Derepression, ribosome reactivation, 311, 312
 Development, sea urchin diagram of, 326
 Diakinesis
 nucleolar morphology, 54
 Triturus oocyte, 54
 "Diffuse" material, in anucleolate nuclei, 152
 Diploid strain cell cultures, nucleolini size in, 168, 171
 Disc electrophoresis
 of nuclear basic proteins, 218
 of nucleolar proteins, 237, 238, 244
 of ribosomal proteins, 237, 238, 244
 DNA
 cistrons
 nucleolar organizer relationship, 564
 565, 567, 568
 for rRNA, 548, 549
 clustering of, 442
 deletion of, 442
 multiplicity in,
 amphibia, 460, 461
 bacteria, 460, 461
 Drosophila, 460, 461
 mammals, 460, 461
 plants, 460, 461
 number in
 chicken, 461
 Drosophila, 451, 452, 453, 454
 as repeats, 461, 462, 463
 variation in number of, 466
 in *Xenopus*, properties of, 441

- DNA—Continued
- cistrons—Continued
 - for tRNA, 441
 - in *Drosophila*, 459
 - in NO region, 456
 - number in *Drosophila*, 459
 - for 18S RNA, 454
 - for 28S RNA, 454
 - complementary to 28S RNA, 436, 437, 438
 - composition of
 - Acetabularia*, 226
 - Asterias*, 226
 - Chironomus*, 226
 - Drosophila*, 226, 455
 - liver nuclei, 194, 195
 - liver nucleolus, 195
 - sea urchin, 329, 330
 - Tegenaria*, 226
 - Triturus*, 226
 - Walker tumor nuclei, 195, 196
 - Walker tumor nucleoli, 195, 196
 - content of
 - isolated nucleoli, 246
 - liver nuclei, 194, 195, 208
 - liver nucleoli, 194, 195
 - plant nucleoli, 68
 - Walker tumor nuclei, 194, 195, 196
 - Walker tumor nucleoli, 195, 196
 - Xenopus*
 - egg, 299
 - liver, 299
 - tadpole, 299
 - filaments, *Smittia* nucleolus, 267
 - metabolic, 461
 - necklace, *Triturus* oocyte, 55, 58, 66
 - nucleolar rings, 66
 - particles, 269
 - preparation of *Xenopus* erythrocyte, 432, 433
 - rings, amphibian oocyte, 461
 - rRNA hybrids, chicken tissue, 462
 - synthesis
 - nucleolus organizer mutant, *Xenopus*, 425, 426
 - relation to nucleolar presence, 312
 - Smittia* salivary gland nucleus, 256
 - Xenopus*, 425, 426
- Doughnut nucleoli of *P. ovata*, 43
- DPN phosphorylase, 286, 287
- DRB (5, 6—dichlororibofuranosylbenzimidazole)
- effect on DNA polymerase transcription, 258, 259
 - inhibition of
 - RNA synthesis, 258, 259
 - uridine incorporation, 258, 259
- Drosophila*, 564, 565, 566
- anucleolate egg development of, 407
 - cistrons for
 - nucleolar RNA, 224
 - ribosomal RNA, 227, 228
 - labeling of nucleolus in, 267
 - nucleolar appearance in, 302
 - nucleolus organizer region in, 183
 - nucleotide composition of
 - cytoplasm, 226
 - DNA, 226, 230
 - nucleolus, 226, 230
 - rRNA in, 147, 152
 - salivary gland nucleolus, DNA particles in, 269
 - spermatocytes, 150, 151, 152
- E**chinoderm oogenesis, nucleolar metabolism in, 311, 312
- "Endomeres", 309
- Endoplasmic reticulum
- effects on
 - actinomycin on liver regeneration, 381
 - amino acid feeding of starved liver cells, 380, 381
 - glucose feeding on starved liver cells, 380
 - in regenerating liver cells, 381, 382
 - in starved liver cells, 380
- Enzymes (*see also* by specific name)
- migration in disc electrophoresis, 253
 - of nucleolus, 204, 247, 285
- Equilibrium gradient centrifugation, 433
- Ergastoplasm, in regenerating liver cells, 381
- Erich ascites carcinoma cells, RNA profiles of, 518, 519
- 8-Ethoxy-caffeine, 413
- Euchromatin, 108
- chromosomes of *P. ovata*, 39, 40
 - uridine incorporation, 19
- Extrachromosomal DNA, amphibian oocyte nucleoli, 57
- Extranucleolar bodies, in maize, 151
- Extranucleolar nuclear RNA
- 18S, 199
 - nucleotide composition of, 201, 202
- Extranucleolar organizers, 153
- F**ast green staining, 350
- basic protein complexes, 217, 218

- Fertilization, sea urchin, 325, 326
- Feulgen-positive material, association with NO, 267, 268
- Feulgen-methylene blue stain, *Allium cepa*, 126
- Feulgen stain, 57, 367
of intranucleolar chromatin, 114
of nucleolonema, 116
- Fibers
argentophilic, 15
100 A
 Allium cepa, 127
 of nucleoli, 127, 520
in nuclear pores, 37
- Fibrillar component
of isolated nucleoli, 571
of nucleoli
 effect of actinomycin on, 209
- Fibrillar zone, *P. ovata*, 43, 44
- Fibrillo-granular component, *Allium cepa* nucleoli, 128
- Fibrils
in anucleolate *Xenopus* nuclei, 151
chromatin, in nucleoli, 128
in cleaving *Triturus*, 151
heterochromatin relationships, 41, 42, 44
in maize microspore nucleoli, 151
in maize nucleoli, 150
in nucleolar body, 50-80, A, 17
in nucleolar vacuoles, 128
in nucleolonema, 14, 15
of pars amorpha, 14, 15, 132
RNA in, 181, 183, 184
 Chironomus, 184
 Sciarids, 184
60-100 A, 68
- Fibrous core, *Triturus* oocyte nucleolus
protein in, 55
RNA in, 55
- Ficus conica*, 92
- Filaments, filamentous component, *see* Fibrils
- Fluorodinitrobenzene, Sanger's reaction, 221
- 5-Fluorouracil
effect on
 nucleolar morphology, 380, 382
 protein labeling in liver cells, 382
 RNA labeling in liver cells, 382
- Formaldehyde
effect on ribosome sedimentation characteristics, 536
fixation of RNP particles, 535, 545
reaction with RNA fraction, 202, 203
- Formation of nucleolus, telophase in plant cells, 36, 69
- Frog nucleoli
DNA content of, 246, 247
protein content of, 246, 247
RNA content of, 246, 247
- FUDR, effect on nucleolar morphology, 170, 171, 312
- Fundulus*, nucleolar appearance in, 302
- Fusion of nucleoli, *Triturus*, 414
- G**astrula
nucleolar appearance in sea urchin, 302, 326, 327
rRNA synthesis in, 312
tRNA synthesis in, 326, 327
- Genetics of nucleolus organizer, *Xenopus*, 412
- Germinal vesicle breakdown, RNA fate, 312
- Giant chromosomes, NO region appearance of, 393, 394, 395, 396
- Glucose, effect on starved liver cell morphology, 380, 381
- Glucose-6-phosphatase, 287
- Glutamic dehydrogenase, 287
- Glycine, labeling in cell cycle, 355, 359
- Glycogen, liver
effect of actinomycin on,
of starved cells
- Granular cortex, *Triturus* oocyte nucleolus
protein in, 55
RNA in, 55
- Granules, *see also* RNP Particles
- Granules
absence in *Xenopus*, 147
Allium cepa, 127
in anucleolate *Xenopus* nuclei, 151
interchromatin, 16, 20
migration of, 36, 37
nuclear RNA containing, 20
nucleolar, 92, 93, 383, 384, 387, 520, 570
 150 A, 17, 20, 125
in nucleolonema, 14, 520
in pars amorpha, 14
perichromatin, 16, 20
 effect of adenosine in tissue culture cells on, 383
in peripheral zone of nucleolus, 150, 151, 183, 184
ribosome-like in nucleolus, 68
RNA, 36
45S-messenger complex in HeLa cells, 153, 154
RNP

- Granules—Continued
 effect of actinomycin on, 209
 relation with 28S RNA, 209, 210, 212
- Grasshopper
 chromosomes
 RNA in, 152
 RNA precursor incorporation into, 351
 spermatogenesis
 cytidine incorporation into RNA, 147
- Growth of nucleolus in *Allium cepa*, 132
- Gryllidae* spermatocytes
 sex chromatin, 77
- Gryllus argentinus*, 77
- Guanase, 285
- Guinea pig liver
 content of nucleoli
 of DNA, 246, 247
 of protein, 246, 247
 of RNA, 246, 247
 effect of puromycin on isolated nucleoli, 214
 isolation of nuclei from, 213, 214
- HeLa** cell
 anisonucleolinos in, 171, 177, 179
 isolation of
 nuclei, 491
 nucleoli, 491
 messenger-granule complex, 153, 154
 nucleoli, 110, 121
- Helianthus annuus*, 73
- Helicosomes, relation to *Peironcito* dictyosomes, 96
- Helix*, nucleolar appearance in, 302
- Hepatectomy
 actinomycin inhibition on liver regeneration, 382
 effect on
 cytoplasmic
 RNA, 370, 371
 volume, 370, 371
 nucleolar
 RNA, 370, 371
 volume, 370, 371
 partial, effects on RNA synthesis, 371, 372
- Heterochromatin
 in chromosomes of *P. ovata*, 40
 fibrillar relationships, 41, 42, 44
 nucleolar connections in *P. ovata*, 40, 44
 nucleolus organizer relationships, 267, 268
 uridine incorporation into, 19
- Histones
 amino acid content of
 liver nuclei, 194, 195, 204, 205
 in liver nucleoli, 194, 195, 204, 205
 in Walker tumor nuclei, 195, 204, 205
 in Walker tumor nucleoli, 195
 chromatography
 calf thymus with sea urchin protein, 327
 effect on nucleolar RNA, 218, 224
 of isolated nuclei, 218
 of isolated nucleoli, 218
 in sea urchin development, 325
- Hutt's disease, nucleoli in, 95
- Hybridization
 of Chironomids, 396
 RNA-DNA, *Xenopus*, 433, 450, 564
 18S RNA with DNA, 436
 28S RNA with DNA, 434
- Hydrogen bonding
 of RNA, 202, 203
 of 45S RNA, 209
- Hypertrophy of nucleoli
 of cancer cells, 21
 of virus infected cells, 21
- Inclusions, ribonucleoprotein in maize, 151
- Informosomes, 542, 570
- Interface precipitin tests, with anti-ribosome serum, 264
- Intranuclear RNP lumps, 564
- Intranucleolar DNA, *Triturus* oocytes, 55
 nucleolar DNA-core attachment, 58
- Intranucleolar membrane, 56, 58
- Ions, effect on nucleolar formation, 416, 417
- Isoalleles, 565
- Isolated nuclei, amino acid incorporation in, 214
- Isolated nucleoli, amino acid incorporation in, 214
- Isolation
 of lampbrush chromosomes
 of nuclei of HeLa cells, 491
 of nucleolar apparatus fraction
 HeLa cells, 492
 L-cells
 liver, 286
 of nucleoli
 from *Asterias* oocytes, 236
 from guinea pig liver, 214
 from rat liver, 286
 from Walker tumor cells, 286
 of ribosomes from *Asterias* oocytes, 236

- Isonucleolinosis
 cell strains exhibiting, 168
 nucleolini size in diploid cells, 168, 171, 176
 tissue culture cells exhibiting, 168
- K**aryosome, in *P. ovata*, 41, 42, 44, 47, 52
- Kidney, effect of thioacetamide on cells of, 378
- Kurnick-Ris stain, 415
- L**actic dehydrogenase, 287
- Lamellate bodies, in maize, 151
- Lampbrush chromosomes
 isolation of, 66
 nucleolus organizer relationship with, 66
 RNA synthesis in, 298
Triturus oocyte, morphology of, 51, 54, 55, 56
 of vertebrate oocytes, 152
- Larval liver, mollusc embryo, 309
- Lathyrus odoratus*, 73
- "Latent organizers," concept of, 184
- Leucine
 alkali-labile incorporation in nucleoli of, 226
 effect of actinomycin on incorporation into starved liver cell protein, 381
 incorporation in cell cycle, 355, 358
 incorporation into protein during sea urchin oogenesis, 315
- Limnaca*, nucleolar appearance in, 302
- Liver
 DNA content of, 195, 208
 glycogen,
 actinomycin effect on amino acid feeding on, 381
 nuclei
 DNA content of, 195, 208
 histone amino acid content of, 195
 protein content of, 194
 RNA composition,
 interphase RNA, 196, 197, 198
 aqueous phase RNA, 196, 197, 198
 RNA content of, 194
 nucleohistones of nucleolus-associated chromatin of, 16
 nucleoli
 actinomycin autoradiograph of, 210
 DNA content of, 194
 histone amino acid content of, 195, 204, 205
 protein content of, 194
 RNA composition of
 aqueous phase RNA, 196, 197, 198
 interphase RNA, 196, 197, 198
 regenerating, nucleolar RNA synthesis in, 203
 RNA in *Xenopus laevis*, 298
 rRNA synthesis in, 300
- Locust, cytidine incorporation in spermatocytes of, 349
- Low temperature
 effect on *Urechis*, 349
 induced accessory nucleoli, 341
- L-protein of *Asterias* nucleoli, 236, 237, 238, 241
- Lysine
 alkali-labile incorporation in nucleoli, 226
 arginine ratio in labeled basic proteins, 327
 incorporation in cell cycle, 355, 357
Lytechinus variegatus, protein synthesis in, 327
- M**agnesium deprivation, effect on amphibian development, 416
- Maize
 microspore nucleoli, 148
 nucleolar fibrils, 150
 nucleolonema, 151
 peripheral nucleolar granules, 150
 translocations, 6L, 6S, 9L, 9S, 148
- Maleic hydrazide, 413
- Malpighian tubule, chromosomes in, 401, 403
- Mammals
 cell culture, ribosome biogenesis of, oocyte, nucleoli of, 18
- Meiosis, RNA synthesis in, 340
- Mercaptopurin, effect of nucleolar morphology, 22
- p*-Mercurochlorobenzoate (*p*-CMB), effect on ribonuclease, 290
- Meristem, of pea nucleolus, 41
- Mesenchyme
 nucleolar appearance in, 302
 sea urchin embryo, 326
- Messenger RNA, *see* RNA, Messenger
- Metachromatic stain, of *Allium cepa* nucleoli, 126, 127, 129
- Metamorphosis, nucleolar appearance in, 302
- Methyl green-pyronine stain, 414
- Methyl thymidine, incorporation in cell cycle, 355

- Methionine incorporation
 effect of RNase during sea urchin oogenesis, 314
 in HeLa cells, 502
 in nucleolus organizer mutant, *Xenopus*, 427, 428
 puromycin inhibition of, 261
 into RNA, 257
 into rRNA, 332, 333
 during sea urchin oogenesis, 314, 315, 316
 into somitic muscle of *Xenopus* embryos, 425, 427, 428
 into tRNA of sea urchin embryos, 331
 transmethylation with RNA, 261
 in *Xenopus* embryos, 427, 428
- Methylation
 cytoplasmic, 335, 336
 of rRNA, 505, 506, 549
 of tRNA, 226
 nuclear, 335
 in *Xenopus*, 427
- Micrococcus lysodeikticus*, RNA polymerase source, 258
- Micronuclei
 nucleolini
 effect of colchicine on, 172, 173
 silver-reducing substances in, 182
 "Micronucleoli," 350, 401, 406
- Microsomes, contamination of isolated nucleoli with, 241, 243
- Microspores, 145, 146, 149, 150, 151
 fibrils in forming nucleoli, 151
 nucleolonema in maize, 151
- Microsporocytes, *Zea mays* cytidine incorporation in, 349
- Migration
 of granules, 36, 37
 of nucleolar RNA, 20
 nucleolar substance, 18
- Minutes mutant*, relationship to tRNA, 459, 460
- Mitochondria, adenosine effect on, 383
- Mitomycin C, effect on nucleolus, 22
- Mitosis
 colchicine effect on, 172, 173
 RNA synthesis in, 337
- Misgrunus*, nucleolar appearance in, 302
- Modulation hypothesis, 334
- Molarity, effect on nucleolar morphology, 55, 56, 57, 66
- Mollusk embryo, nucleolus in, 309
- Monkey kidney cells
 morphology of, 383, 387
 nucleoli
 effect of adenosine on, 387, 388
- Mouse
 nucleolar appearance in, 302
 RNA synthesis in nucleoli of, 308
 thymidine incorporation in nucleoli, 19
- Mutations, inhibition of nucleolus formation, 445, 446
- Myogryllus verticalis*, 77
- Nebennukleolen, 406
- Neoplastic cells, nucleolini in, 172, 173
- Neurula, nucleolar appearance in, 302
- Nigrella aruensis*, RNA synthesis in, 339, 346, 347
- Nissl bodies, 407
- Nitroquinoline-*N*-oxide, 22
 effect on nucleolini, 171
 effect on nucleolar morphology, 170, 172
- Nogalomycin, 22
- Nucleohistone
 cytochemical demonstration of, 16
 of liver, 16
 of nucleolus-associated chromatin, 15, 16
- Nucleolini, 15, 167, 168
 Balbiani ring relationship to, 180
 chromosome number relationship to, 173
 differences
 in aneuploid cells, 172, 173
 in euploid cells, 171
 effect
 of carcinogens on, 173
 of chemicals on, 170, 171
 of colchicine on, 172
 of metabolites on, 173
 of viruses on, 367
 in neoplastic cells, 172, 173
 size in diploid strains, 171
- Nucleolonema, 36, 37, 105, 571
 in *Allium cepa*, 127, 128
 argentiphilic substances in, 14, 15
 in *Armadillidium nasatum*, 100, 101, 102
 ATPase in, 94
 in cat, 100
 in chicken fibroblasts, 112, 113
 definition of, 107
 demonstration of, 92
 dynamics of, 91
 effect of adenosine on, 383, 388, 389
 fibrils in, 14, 15
 in fresh material, 92
 granules, 96
 growth, 14, 15, 520
 in HeLa cells, 120, 121

Nucleolonema—Continued

- loops in, 112, 120, 121
- in maize microspores, 151
- during mitosis, 95
- in monkey kidney cells, 383, 386, 387
- morphology of, 91
- in *Omexechae servilli*, 101
- origin of, 95
- relationships to nucleolus organizer, 96, 97
- ribbon-like, 112, 113, 114
- self-replication, 96
- of spinal cord, 103
- staining procedures for, 111, 120, 121
- uridine incorporation in, 19

Nucleolus (species list)

- of *Acetabularia*, 226
- of *Acricotopus lucidus* salivary gland cell, 408, 409
- of *Allium cepa* root tip, 136, 137, 138, 139, 140, 141, 188, 189, 337
- of *Amblystoma mexicanum* oocyte, 475
- of *Arbacia*
 - embryo, 311
 - oocyte, 311
- of *Armadillidium nasatum*
 - oocyte, 100
 - salivary gland, 100, 101
- of *Ascidia*, 302
- of *Asterias* oocyte, 223, 235, 311
- of cancer cell, 21
 - Pomerat's, 178, 179
- of chicken, 302
 - fibroblast, 118, 119
- of Chironomids, 181, 255, 393
- of *Chironomus*
 - malpighian tubule, 400, 401, 402, 403
 - salivary gland, 226
- of *Chiton*, 302
- of chondrocyte, NO mutant, *Xenopus*, 420, 421
- of *Chortophaga*, 43
- of *Ciona*, 302
- of CMP epithelial adenocarcinoma, 353
- of *Coregonus*, 302
- of Corn microsporocytes, 348
- of *Cyclops*, 302
- of *Drosophila* salivary gland, 226
- of eggs, *Urechis*, 346
- of frog, 302
 - oocyte, 246, 249
- of *Fundulus*, 302
- of *Gryllus* spermatocyte, 77
- of guinea pig liver, 213, 246
- of HeLa cells, 16, 118, 176, 489
- of *Helianthus* meristem, k73

- of *Helix*, 302
- of HEP #2 cell, 176
- in Hutt's disease, 95
- of intestinal epithelium, 176
- of kidney, monkey, 19, 32, 276, 380, 386
- of *Lathyrus odoratus* meristem, 72
- of L-cells, 527
- of leukemia cell, 176
- of *Limnaea*, 302
- of liver
 - regenerating, rat, 369, 379
 - rat, 193, 235, 285, 297, 379
 - Xenopus*, NO mutant, 420
- of locust spermatocyte, 348
- of *Lytechinus* embryo, 325
- of maize microspores, 158
- of malpighian tubule, *Chironomus*, 400
- of meristem
 - Allium cepa*, 136, 188, 337
 - Helianthus*, 69, 72
 - Lathyrus odoratus*, 69
 - Plantago ovata*, 40, 46
 - Raphanus sativus*, 72
 - Tropaeolum majus*, 72
 - Vicia faba*, 271
- of microsporocytes, corn, 348
- of *Misgrunus*, 302
- of mollusk embryo, 309
- of monkey kidney, 19, 32, 176, 380, 387
- of mouse, 302
- of muscle, *Xenopus* NO mutant, 420
- of *Myogryllus verticalis* spermatocyte, 77
- of nerve cell, 14
- of *Nigrella orvensis*, 346
- of nucleolus organizer mutant, *Xenopus*, 297, 411, 425, 431
- of *Omexechae servillei* Blanch. spermatocyte, 101
- of oocyte
 - Amblystoma*, 145, 475
 - Armadillidium*, 100
 - Asterias*, 226, 235, 311
 - frog, 246
 - Paracentrotus*, 311
 - Plethodon*, 57
 - sea urchin, 311
 - spider, 246
 - Tegenaria*, 226
 - Triturus*, 53, 145, 226, 475
 - Urechis*, 346
 - Xenopus*, 297, 431
- of pancreas, rat, 16, 28
- of *Paracentrotus*, 311
- of pea seedling, 246
- Plantago ovata*, meristem, 40, 46

- Nucleolus—Continued
- of Pomerat's carcinoma, 178
 - of pronephric cell, NO mutant, *Xenopus* embryo, 420
 - of *Raphanus sativus* meristem, 72
 - of rat
 - liver, 16, 34, 246, 285, 302, 380, 386
 - pancreas, 16, 28
 - regenerating liver, 368, 379
 - of *Rhynchosciara*, 36, 268, 406, 447, 559
 - of salamander oocyte
 - Amblystoma*, 145, 475
 - Triturus*, 53, 145, 226, 475
 - of salivary gland
 - Acricotopus*, 400
 - of *Armadillidium*, 100
 - Chironomus*, 226, 400
 - Drosophila*, 226
 - of *Smittia*, 182, 255
 - of *Schistocerca gregaria* (locust) spermatocyte, 348
 - of sea urchin, 302
 - Lytechinus* embryo, 325
 - of sea urchin oocyte
 - Arbacia*, 311
 - Paracentrotus*, 311
 - of *Smittia* salivary gland, 182, 255
 - of spermatocytes
 - Gryllus*, 77
 - locust (*Schistocerca*), 348
 - Myogryllus*, 77
 - Omexechae servillei*, Blanch., 101
 - Urechis*, 348
 - of spider, 246
 - of spinal cord, 103
 - of SV40 cells, 21, 176
 - of *Tegenaria*, 226
 - of *Triturus* oocytes, 53, 145, 226, 302, 475
 - of *Tropocolum majus*, 72
 - of *Urechis*
 - male reproductive cells, 181, 193, 337
 - oocyte, 346
 - of *Vicia faba* meristem, 271
 - of virus-infected cells, 21
 - of Walker tumor cells, 193, 246
 - of WI-38 cells, 176
 - of *Xenopus*, 302
 - embryos, 297, 425, 431
 - nucleolus organizer mutant, 297, 431
 - chondrocyte, 420
 - liver, 420
 - muscle, 420
 - pronephric cell, 420
 - oocyte, 297, 431
 - of *Zea mays*, microsporocytes, 348
 - apparatus fraction, isolation of
 - from HeLa cells
 - from "L" cells
 - from liver cells
 - associated chromatin, 108, 571
 - in rat liver nucleoli, 380
 - thymidine incorporation in, 19
 - attachment to chromosomes, 394
 - chromosome relationships, 559
 - in *Gryllidae*, 84
 - composition of, 564, 570
 - definition of, 563, 564
 - DNA
 - in amphibian oocyte, 224
 - attachment to intranucleolar membrane, 58
 - composition in liver, 195
 - EM studies on, 567
 - relation to rRNA cistrons, 571
 - of *Smittia*, 263, 264
 - thymidine incorporation in, 37
 - effect of phenol on, 514, 515
 - fibrils, 520
 - effect of DNase on, 56
 - effect of RNase on, 56
 - effect of trypsin on, 56
 - formation of, 416
 - genes, 528
 - granules, 166, 520
 - in pyramidal cells, 93
 - growth, influence of organizer, 132
 - heterochromatin connection, 40, 44
 - isolation of
 - from *Asterias*, 236
 - from guinea pig liver, 214
 - from rat liver, 286
 - from Walker tumor cells, 286
 - knobs in *Smittia*, 268
 - methionine incorporation in, 261
 - morphology
 - components of, 570
 - cytochemistry of, 15
 - effects
 - of actinomycin on, 22, 170, 171, 380
 - of adenosine on, 380, 383
 - of aflatoxin on, 22, 23
 - of aminopterin on, 22
 - of azaguanine on, 22
 - of azaserine on, 22
 - of canavanine on, 22
 - of chloramphenicol on, 22
 - of colchicine on, 170, 171
 - of diet on, 170, 171
 - of fixation on, 15, 379, 380
 - of 5-fluorouracil on, 380, 382
 - of FUDR on, 170, 171

- Nucleolus—Continued
 morphology—Continued
 effects—Continued
 of mercaptopurine on, 22
 of mitomycin on, 22
 of molarity on, 55, 56
 of nitroquinoline-N-oxide on, 22, 170, 171
 of nogalomyacin on, 22
 of purine analogues on, 22
 of puromycin on, 22
 of pyrimidine analogues on, 22
 of thioacetamide on, 23, 370
 of ultraviolet irradiation on, 22
 of *Vinca* alkaloids on, 22
 of virus infection on, 367
 X-irradiation, 22
 of *P. ovata*, 43, 47
 of *Smittia*, 256, 268
 of tissue culture cells, 107
 of *Triturus* oocytes, 54, 55
 of *Urechis*, 181, 337
 organizer, 110, 145, 183, 446
 of *Allium cepa*, 131
 argentiphilic material in, 181
 association with "bobbed" locus, 464
 of *Chironomus*, 53, 67, 107, 224, 393
 chromatin effects in plants, 227
 definition of, 567
 effect on nucleolar number, 413, 414
 function of, 566
 of giant chromosomes, 393, 394
 of lampbrush chromosomes, 66
 of maize, 446
 mutant of *Xenopus laevis*, 432
 cytology of, 414
 DNA synthesis in, 425, 426
 effect on cytoplasmic RNA, 115
 effect on nuclear size, 301, 302, 413
 effect on nucleolar morphology, 301, 302, 416
 hybrid formation of RNA-DNA, 436, 437
 methionine incorporation in, 428
 protein synthesis in, 428
 RNA synthesis in, 426
 rRNA synthesis in, 301, 302, 426
 tRNA synthesis in, 302, 445
 uridine incorporation in, 426, 427
 in *P. ovata*, 39, 40, 43
 relation to nucleolar activity, 395, 396
 in *Rhynchosciara*, 268
 rRNA cistrons location, 57, 445
 role in nucleolus accumulation, 147
 synthetic activity of, 131
 in telophase of *Allium cepa*, 129
 X-ray fragmentation of, 397, 407
 P³² incorporation in,
 protein (*see also* Proteins)
 amino acid incorporation into, 153
 of *Asterias*, 235
 fractionation of, 216, 217, 236
 nonribosome-like, 570
 residual, ribosome-like, 570
 synthesis, site of, 153
 of *Triturus* oocyte, 55
 rings of DNA, 55, 58, 66
 RNA (*see* Ribonucleic acid)
 size
 in cell culture,
 in *P. ovata*, 41
 in *Xenopus*, 110, 422
 somatic, 298
 in telophase of *Allium cepa*,
 time of appearance
 in *Ascidia*, 302
 in chicken, 302
 in *Chironomus*, 302
 in *Chiton*, 302
 in *Ciona*, 302
 in *Coregonus*, 302
 in *Cyclops*, 302
 in development, 302
 in *Drosophila*, 302
 in *Fundulus*, 302
 in *Helix*, 302
 in *Limnaea*, 302
 in *Misgrunus*, 302
 in mouse, 302
 in *Rana*, 302
 in rat, 302
 in sea urchin, 302, 326
 in *Triturus*, 302
 in *Xenopus*, 302
 uridine incorporation
 in *Chironomus*, 395, 400
 in *Xenopus*, 426
 vacuoles, 92
 Nucleoplasm, *see* Nuclear Sap
 Nucleosides, autoradiography with, 298
 Nucleoside phosphorylase, 285
 5'Nucleotidase, 287
 Nucleotide composition, *see* RNA, DNA
 Composition
 Nucleus
 amino acid incorporation in, 215
 DNA composition in rat liver, 195
 fractionation of RNA in hot phenol, 514, 515

- Nucleus—Continued
 membrane of
 relationship to nucleolus, 16
 pores, 18, 36, 37
 fibers in, 37
 residue, 514
 RNA (*see* Ribonucleic Acid)
 sap
 ribosomes of, 513, 514
 RNA precursors in, 314, 315
 of tissue culture cells, 383, 386, 387
 transplantation of, 303
- Omecechaes serville*, Blanch., nucleolonema of, 101
- Onion, RNA on chromosomes of, 152
- Oocytes
 amphibian, 54
Asterias, 235, 311
 cytidine incorporation in of sea urchin, 314, 315, 321, 322
 DNA content of *Xenopus*, 299
 in meiosis, 300
 nucleolar fate in, 311
 pachytene nucleoli of *Triturus*, 54
Plethodon, 58
 protein synthesis of sea urchin, 314
 RNA
 content of *Xenopus*, 300
 mRNA linkage to ribosomes of, 315
 rRNA synthesis in *Xenopus*, 298
 synthesis in
 Ophryotrocha, 341
 Paracentrotus, 313, 315, 320
 uridine incorporation in of sea urchin, 315
- Oogenesis
 nucleolar metabolism in Echinoderms, 311
 nucleolar RNA activity in, 312
 rRNA synthesis in, 298, 300
- Ophryotrocha*, RNA synthesis in oocyte of, 341
- Origin of nucleolar materials, *Allium cepa*, 132
- Orthophosphate
 lead precipitate of, 194
 in nucleoli, 181
- Pachytene nucleoli of *Triturus* oocyte, morphology of, 54
- Pancreas, nucleohistone of nucleolus associated chromatin, 16
- Paracentrotus lividus* oocyte, RNA synthesis in, 312
- Paranucleoli, nucleolini relationship, 180
- Pars amorpha, 41, 92, 571
 in *Allium cepa*, 128, 129
 in *Armillidium nasatum*, 102
 definition of, 94
 dissolution of, 131
 effect of adenosine in tissue culture cells, 383
 fibrils, 15
 granules, 15
 in HeLa cells, 111, 121
 in monkey kidney cells, 383, 387
 nucleolonema relationship, 94
 RNP demonstration of, 168
 of starved rat liver cells, 380
 in telophase nucleoli of *Allium cepa*, 132
 vacuoles in, 135
- Pathology of nucleoli, 94
- Particles, *see* Granules
- Pea seedling nucleoli
 DNA content of, 246
 isolation of, 214
 protein content of, 246
 puromycin effect on, 214
 ribosomal DNA cistron localization of, 227
- RNA
 content of, 246
 synthesis in, 227
 RNP particles in, 210
 60S particles, 210
- "Perinucleolar network"
 rat liver nucleoli demonstration of, 209
 Walker tumor nucleoli demonstration of, 209
- Perinucleolar structures, 571
- Phenocopy, of nucleolus deficiency, 416
- Phenolic nuclei, 514, 515
- P³² incorporation into
 ascites cells, 37
Asterias ribosomes, 241, 242
 liver nuclear 18S RNA, 199, 200, 208
 liver nucleolar 18S RNA, 199
 nucleolar DNA, 37
 rootlets, 190
 root tips, 182
 sea urchin embryo RNA, 330, 331
Xenopus
 liver rRNA, 299
 oocyte rRNA, 300
- Phosphatidopeptides, 190

- Plantago ovata*
 nucleolus
 fine structure of
 fibrillar zone, 42, 44
 ribosomal cortex, 43, 47
 ribosomal zone, 43, 47
 size of, 41
 volume of, 40, 41
 root tip meristem fine structure of, 40, 47, 49, 51
- Plethodon* oocyte
 nucleolar extrachromosomal DNA of, 57
 ring nucleoli of, 58, 66
- Pluteus stage of Echinoderm embryo
 protein synthesis in, 327
 rRNA synthesis initiation in, 326
- Polysomes of monkey kidney cells, aden-
 osine effect on, 383
- Polyvinylsulfate in RNA extraction, 239
- Prenucleolar bodies, 184, 350
 origin of, 350
 silver staining of, 350
- Prenucleolar material, metachromatic, 69
 "Preribosomal" RNA, 376
- Prochromosomes of *Urechis*, 338
- Proflavine, effect on nucleolar morphol-
 ogy, 22
- Prophase nucleolus, 337
 in *Allium cepa*, RNA synthesis in, 131
- Pronephron, RNA increase in *Xenopus*
 mutant, 422
- Protein(s)
 acidic of nucleoli, 204
 amino acid content of, 205
 amounts in isolated nucleoli, 246
 antigenic properties of
 nucleolar, 238
 ribosomal, 238
 of *Asterias* nucleoli, 236
 autoradiography of *Smittia* salivary
 gland nuclear, 256
 basic
 alanine incorporation into, 327
 amino acid incorporation into, 216
 of *Asterias* nucleoli, 236, 246
 of guinea pig liver, 246
 lysine-arginine ratio of, 329
 of nucleolus (*see* residue), 217, 218
 of nucleus, 216, 217
 synthesis during sea urchin develop-
 ment, 327
 common between nucleolus and ribo-
 somes, 244, 251
 content of
 liver nuclei, 194
 liver nucleoli, 194
 Walker tumor nuclei, 194
 Walker tumor nucleoli, 194
 differences between nucleolar and ribo-
 somal, 243, 251
 disc electrophoresis of
 nucleolar, 243
 nucleolar basic, 218
 ribosomal, 243
 end groups of *Asterias* nucleoli, 236
 extraction of
 nucleolar, 237
 ribosomal, 237
 fractionation of
 nuclear, 216, 217
 nucleolar, 216, 217
 of lampbrush loops, 559
 L-protein of nucleolus, 236
 lysine rich of nucleolus, 227
 migration on acrylamide gels of, 243,
 251
 nascent, 252
 removal from ribosomes, 252
 on ribosomes, 252
 nonbasic
 of *Asterias* nucleolus, 247
 of guinea pig liver, 247
 of pea seedlings, 216, 247
 nonhistone in puffs, 153
 nuclear, disc electrophoresis of, 218
 of nucleolus, 559
 classes of, 205, 210
 at organizer, 447
 starch gel electrophoresis of, 210
 synthesis, site of, 153
 preparation of antibodies to, 237
 nucleolar, 237
 ribosomal, 237
 profiles of sea urchin embryo, 328
 of puffs, 559
 residue,
 amino acid incorporation into, 216
 of nucleolus, 217, 218
 of *Asterias*, 236
 of pea seedlings, 215
 of nucleus, 216, 217
 ribosomal
 of nucleolus, 153
 number of species of, 551
 synthesis of, 551, 552
 site of, 551

- Protein(s)—Continued
- starvation
 - effect on ribosomes, 390
 - synthesis
 - effect of
 - actinomycin in regenerating liver cell, 382
 - 5-fluorouracil on liver cell, 382
 - leucine incorporation in sea urchin oocytes, 315
 - methionine incorporation in sea urchin oocytes, 314
 - nuclear in thymocytes, 224
 - in nucleoli, 520
 - puromycin inhibition of, 428
 - in regenerating liver cells, 380
 - stages in sea urchin embryo of, 326
- Prototroch, nucleolar appearance in, 302
- Pseudouridine incorporation
 - into nucleolar 4S RNA, 226
 - into "t-like" RNA, 336
- Puffs, 166, 559
 - chromosomal, 394
 - formation of, 153
- Purine analogues, effect on nucleolar morphology, 22
- Puromycin effect on
 - cleavage, 311
 - isolated guinea pig nucleoli, 214
 - isolated pea nucleoli, 214
 - methionine incorporation, 257, 261, 428
 - on nucleolar morphology, 22
- Pyrimidine analogue, effect on nucleolar morphology, 22
- Pyronine stain, *Xenopus* blastula, 308
- Pyruvate kinase, 287
- Q**uasidiploid cells, nucleolini differences, 172
- Quiescent zone nucleoli, *P. ovata*, 41, 47
- R**ana, nucleolar appearance in, 302
- Raphanus sativus*, 73
- Rat liver
 - nuclei
 - effect of protein-free diet on, 379
 - effect of protein-rich diet on, 379
 - effect of starvation on, 387
 - isolation in cold phenol, 514
 - isolation of nucleoli of, 514, 525
 - rRNA precursors of, 514
 - nucleoli
 - appearance of, 302
 - DNA content of, 246, 247
 - isolation of, 194
 - protein content of, 246, 247
 - RNA content of, 246, 247
 - polyribosomes of, 533
 - regeneration
 - composition of nuclear RNA, 195, 198
 - composition of nucleolar RNA, 195, 198
 - RNA synthesis in, 381
 - turnover of 45S RNA in, 203
- Regulation of rRNA synthesis, 299, 303
- Residue protein of *Asterias nucleoli*, 236, 238
- Rhynchosciara*, 36, 447, 559
 - micronucleoli of, 406, 409
 - nucleolus organizer of, 268
- Ribonuclease, 285, 287, 288, 289
 - digestion of nucleolar RNA, 17
 - effect of
 - actinomycin on, 289
 - p-CMB on, 289, 290
 - thioacetamide on, 289, 290, 293, 376
 - Triton X-100 on, 289, 290
 - methionine incorporation, 314
 - nucleolar morphology, 56
 - of liver nucleolus, 204, 250
- Ribonucleic acid (RNA)
- 4S RNA, *see* Transfer RNA
- 6S RNA
 - composition in rat liver nucleoli of, 208
 - nucleolar origin of, 209
- 16S RNA, *see* 18S RNA
- 18S RNA, 183, 206, 547, 548, 549, 555, 556, 559
 - absence as measure of nuclear purity, 512
 - of *Asterias nucleoli*, 238, 240, 246
 - of *Asterias* ribosome, 238, 239, 240
 - of *Chironomus*, 488
 - cistrons for, 454
 - in *Drosophila*, 565
 - in *Xenopus*, 565
 - composition in salamander oocytes, 478
 - as contaminant of isolated nuclei, 503, 512
 - formation of, 210
 - in HeLa cells, 493
 - hybrid formation with NO-DNA, 436
 - in liver cytoplasm, 482
 - in liver nuclei, 199
 - in liver nucleoli, 199
 - of nucleolus, 567
 - in *Xenopus*, 152

- 28S RNA, 183, 206, 547, 548, 549, 555, 556, 559, 560
 of *Asterias* nucleolus, 238, 246, 247
 of *Asterias* ribosome, 238
 of *Chironomus*, 488
 cistrons for
 in *Drosophila*, 565
 in erythrocytes, 438
 in tadpoles, 438
 in *Xenopus*, 436
 composition in rat liver nucleoli, 208
 composition in salamander oocytes, 478
 effect of thioacetamide on, 378
 formation in nucleolus, 518
 of HeLa cells, 493, 501
 hybrid formation with NO-DNA, 437
 of nucleolus, 567
 of ribonucleoprotein granules, 209
 of *Smittia* salivary gland, 259
 of *Xenopus*, 152, 257, 434
- 30-35S RNA, 547, 548, 549, 555, 556, 559, 590
 composition in rat liver nucleoli, 208
 conversion of, 551, 553
 effect of thioacetamide on in rat liver, 199
 extranucleolar-nuclear of rat liver, 199
 of HeLa cells, 494
 nuclear localization of, 484
 of nucleolus, 567
 of rat liver nucleolus, 199, 516, 517
 of rat liver nucleus, 199, 516, 517
 synthesis in nucleolus, 518
- 40-45S RNA
 absence in bacteria, 569
 absence in plants, 569
 cleavage in nucleoli, 505
 composition in rat liver of, 208
 DNA-like, 569
 effect of thioacetamide on
 nuclear, 199
 nucleolar, 199
 extranucleolar-nuclear, 199
 in HeLa cells, 493
 heterogeneity of, 488
 hydrogen bonding of, 209
 nuclear
 effect of thioacetamide on, 199
 turnover time of, 203
 nucleolar, 199, 547, 548, 549, 552, 555, 558, 559, 560, 567
 localization of, 484
 transformation of, 529, 542
 turnover time of, 203
 of rat liver nuclei, 199, 515, 516
 synthesis in nucleoli of, 505
 of *Triturus* oocyte nucleolus, 57
 turnover in Walker tumor nucleolus, 203
- 55S RNA
 effect of thioacetamide on
 nuclear, 199
 nucleolar, 199
- RNA
 "A" (aqueous)
 bodies, 350
 chromosomal
 effects of benzimidazoles on, 226
 of "puffs," 153
 synthesis in lampbrush chromosomes, 484, 487
 composition of
 Balbiani rings, 230
 cytoplasm, in
 Acetabularia, 226
 Asterias, 226
 Chironomus, 226
 Drosophila, 226
 sea urchin embryos, 226
 Tegenaria, 330
 Triturus, 226
 Xenopus, 226
 nucleolus, in
 Acetabularia, 226
 Asterias, 226, 251
 Chironomus, 226
 Drosophila, 226
 normal rat liver, 198, 201, 208
 regenerating rat liver, 198
Tegenaria, 226
Triturus, 57, 226, 478
 Walker tumor cells, 108
 nucleus, in
 normal rat liver, 198, 514, 517
 regenerating rat liver, 198
 Walker tumor cells, 197
 tRNA of *Drosophila*, 455
Xenopus, 445
- content
 after hepatectomy, 371
 in isolated nucleoli, 246
 in liver nuclei, 194
 in liver nucleoli, 194
 in *Triturus* oocytes, 475
 in Walker tumor nuclei, 195
 in Walker tumor nucleoli, 195
- cytoplasmic
 effect of NO⁻ mutant on, 426

- RNA—Continued
cytoplasmic—Continued
 in HeLa cells, 494, 495, 499
 nucleotide composition of
 in *Acetabularia*, 226
 in *Asterias*, 226
 in *Chironomus*, 226
 in *Drosophila*, 226
 in *Tegenaria*, 226
 in *Triturus*, 226
 relation to nucleolar, 224
 uridine incorporation in oocyte, 478, 482
differences in nucleolar, 181
-DNA hybrid formation, 433, 436, 445, 446, 517, 541
extranucleolar, 201, 569
fibrillar, 184
 in *Chironomus*, 184
 in nucleoli, 181, 184
 in Sciarids, 184
formaldehyde reaction with, 202
fractionation of
 mouse nucleoli, 153
 in nucleoli, 153
 with phenol, 514
 in *Vicia faba*, 272
gel filtration of, 272
hydrogen bonding of, 203
"I" (interphase), 196, 197
incorporation of precursors
 adenylic acid, 258
 effect of actinomycin on, 266
 effect of 5-fluorouracil on, 382
 pseudouridine, 226
 in sea urchin oocytes, 315
 in *Triturus* oocytes, 475
loss from tissue sections, 282
messenger (mRNA)
(messenger RNA, dRNA, DNA-like RNA)
 actinomycin sensitivity, 514
 aggregation with 18S RNA, 480
 aggregation with 30S RNP particle, 421, 520, 522
 aggregation with 40S RNP particle, 535
 of ascites cells, 517
 attachment to ribosomes in sea urchin, 315
 contamination of rRNA, 472
 in embryos with transplanted nuclei, 309
 synthesis
 in *Asterias* oocytes, 312
 in early embryos, 304
 in rat liver, 514, 516
 in sea urchin embryos, 326, 334
 methylase, 286
 methylation
 effect of actinomycin on, 263
 effect of benzimidazole, 264
 in sea urchin gastrula, 329
 of tRNA, 226
 transmethylation in nucleolus, 257, 261, 263
nuclear
 in HeLa cells, 493, 494, 495
 in nucleoplasm, 483, 484, 487, 488
 phenol extraction of, 491
 relation to cell size, 225
 uridine incorporation in oocytes of, 479, 480, 481
nucleolar, *see also* 45S, 28S, 18S, tRNA
 DNA cistrons for, 224
 effect of histone on, 224
 fibrillar, 17
 granular, 16
 migration of, 19, 20
 relationships to cytoplasmic, 224, 225, 226
 synthesis in viral infection, 21, 22
 terminal addition of adenine, 226
 terminal addition of cytosine, 226
polymerase, 285, 286, 287, 292
 effect of actinomycin on, 390
 effect of DRB on, 258, 259
 effect of thioacetamide on, 376
 effect of TRB on, 258, 259
 of rat liver nucleolus, 204
profiles
 actinomycin inhibition in rat liver, 381, 382
 actinomycin inhibition in *Smittia*, 261, 262
 of *Asterias* nucleoli, 238, 239
 of *Asterias* ribosomes, 238, 239
 of Ehrlich ascites carcinoma cells, 518, 519
 of HeLa cell nuclei, 489, 493, 494, 499, 508, 509, 510, 511
 of "L" cells, 530, 531
 of rat liver, 199, 201, 381, 382
 of rat liver nuclei, 515, 516
 of RNA methylation,
 of *Smittia* salivary glands, 256, 257, 259, 260
 of *Triturus* oocytes, 479, 480, 481, 482
 of Walker tumor cells, 199, 201

RNA—Continued

- ribosomal (rRNA), *see also* 18S, 28S, 30-35S, 45S RNA
 - cistrons, *see also* DNA Cistrons of rRNA
 - deletions in *Xenopus*, 422
 - in nucleolus organizer, 57
 - relation to "bobbed" mutant, 462, 471, 472
- DNA hybrids, 184
 - in *Drosophila*, 455
- in *Drosophila*, 152
- as message for ribosomal protein, 551
- methylation of, 548, 549
- in nucleoli, 224, 225, 226
- P³² incorporation
 - in *Asterias*, 235
 - in sea urchin, 332
- precursor, 500, 501
 - extraction of, 514
 - nucleolar site of, 514, 515
 - in rat liver nuclei, 513
- synthesis of, 447
 - in HeLa cells, 503
 - in NO mutant, 515
 - in nucleolus, 230, 299, 426, 429, 430
 - in relation to nucleolar appearance, 312
 - in sea urchin, 312, 326, 332, 333
 - in *Smittia*, 259
 - in *Xenopus* oocytes, 299, 300
- transfer to cytoplasm, 504
- of *Xenopus*, 226, 227
- synthesis
 - in *Arbacia* oogenesis, 312
 - during cell cycle, 190
 - effect of actinomycin on regenerating liver, 381
 - in lampbrush chromosomes, 298
 - in nucleolus, 146, 147
 - of *Chironomus*, 13
 - effect of actinomycin on, 226, 227
 - in isolated, 214, 236, 236
 - of NO⁻ mutant, 425
 - of peas, 227
 - of *Triturus*, 20, 57
- at nucleolus organizer, 147
- in regenerating liver, 203, 381
- in root tips of *Nigrella*, 13
- in sea urchin cleavage, 325, 326
- in *Smittia* salivary glands, 256, 257, 258
- in *Xenopus*, 425, 426
- transfer (tRNA, 4S RNA)
 - actinomycin effect, 266
 - aminoacylleucyl-, 273

- of *Asterias*, 225, 236, 246
- attachment to rRNA, 335
- of *Chironomus*, 255
- cistrons, 459, 528
- composition in salamander oocytes, 478
- C-C-A turnover, 282
- cytidine incorporation, 315
- DNA hybrid formation in *Drosophila*, 456
- extraction with phenol, 272
- methionine incorporation in, 332
- methylation of, 261, 263, 335, 427, 569
- of nucleoli, 221, 225, 228, 264, 450
- P³² incorporation in, 337
- precursor incorporation in, 267
- ribothymidine in, 264
- in salamander oocytes, 475
- synthesis of, 226, 261, 263, 307, 528
 - in embryo, 301
 - in sea urchin embryo, 326, 329, 331
 - in *Smittia*, 226, 255
- uridine incorporation, 259, 478, 480, 481
- in *Vicia faba*, 272, 275, 569
- in *Xenopus laevis*, 299
- Ribonucleoprotein (RNP)
 - network, 212, 547
 - nuclear organization, 519, 520
 - particles, *see also* Granules
 - particles, 406
 - in maize, 151
 - messenger association with, 520, 521, 522
 - of nucleolus, 519, 520, 554
 - in pea nucleoli, 210, 550, 551
 - TBM demonstration of, 167, 168
- 40S, 537
 - buoyant density analysis of, 534
 - cytidine incorporation into, 430, 531, 532, 540
 - cytoplasmic appearance of, 529, 530, 532
 - function of, 541
- 60S, 206
 - buoyant density analysis of, 533, 536, 537
 - cytidine incorporation into, 530, 531, 532, 544
 - cytoplasmic appearance of, 529, 530, 532
 - of pea nucleoli, 210
- 76S, 529, 530, 533
- profiles of, 522, 530

- Ribosome(s)
 activity
 in sea urchin eggs, 311, 312
 after trypsin, 312
 antigens to nucleolar proteins, 238
 assembly, site of, 547
 biogenesis of, 547
 clumps in nucleoli of *P. ovata*, 43
 contamination of isolated nucleoli, 242
 cortex in *P. ovata*, 43, 46, 47
 cytoplasmic
 effect of adenosine on, 383
 effect of protein starvation on, 390
 labeling in sea urchin development
 of, 328
 origin of, 36, 37
 P³² incorporation in *Asterias*, 241
 uridine incorporation in *Triturus*,
 483
 in developing sea urchin nucleus, 327
 DNA cistrons
 in *Drosophila*, 227
 in pea seedlings, 227
 in *Xenopus*, 227
 formaldehyde effects on, 536
 formation, nucleolar involvement, 528,
 529
 nuclear, accumulation of, 483
 in nucleoli, *see also* Granules, 519, 520
 precursors
 conversion of, 483, 484, 485
 in HeLa cells, 503, 504
 in nucleoli, 520
 protein
 antibodies to, 235
 extraction of, 237
 gel electrophoresis of, 235
 nucleolar protein relationship to, 235
 synthesis, site of, 551
 region of nucleoli, 43
 RNA profiles of *Asterias*, 238, 239, 240
 storage in oocytes of, 304
 subunits
 effect of EDTA on, 55
 proteins of 50S, 552
 tRNA attachment to, 333, 335
 uridine incorporation into, 533, 534,
 535
 60S structure of, 550, 554, 559
 zone of *P. ovata*, 43, 46, 47
 Ribothymidine
 in nucleolar RNA, 264
 from uridine, 264
 Ring nucleoli, 58, 66
 Roncaroni body, 95
 Root tips
 amino acid incorporation in nucleoli of,
 153
 P³² incorporation in, 182, 183
 RNA synthesis, 182, 337
 in *Allium cepa*, 339
 in *Nigrella oriensis*, 346, 347
*S*alamander, *see Trituris, Amblystoma*
 Salivary glands
 Drosophila, 147
 P³² pattern in, 182, 183
 puffs of, 146, 152
 rRNA synthesis in polytene chromo-
 somes of, 447
 silver-reducing substances in *Smittia*,
 187
Schistocerca gregonia, RNA synthesis in,
 337
Sciara
 fibrillar RNA component of, 184
 nucleolus organizer heterochromatin
 relationship, 267
 Scute 4-Scute 8 mutant
 chromosome breakage points, 565
 relationship to rRNA cistrons, 463
 Sea urchin
 embryo
 stages of protein synthesis in, 325,
 326
 oocyte
 methionine incorporation in, 314, 315,
 316
 nucleolar appearance in, 302, 306
 Sedimentation coefficients, salamander
 ovarian RNA, 477, 478
 Segregation of nuclear components, ac-
 tinomycin effects, 22
 Sephadex, gel filtration of RNA, 272, 273
 Sex chromosomes, *Gryllidae* 78, 79
 Silver-reducing substances
 in micronuclei, 182
 in nucleolus organizer, 181
 in *Smittia* salivary glands, 182
 Skin tests, for common ribosomal and
 nucleolar antigens, 238
Smittia
 breeding stages of, 255, 256
 salivary gland nucleolus
 autoradiography of, 267, 268
 labeling in, 267
 morphology of, 268
 tRNA synthesis in, 255
 silver-reducing substances in, 182
 uridine incorporation in larvae, 259,
 260

- Snake venom diesterase, 266
- Sonication
effect on
 amino acid incorporation in isolated nucleoli, 214, 215, 221
 DNA content
 of isolated nuclei, 194, 195
 of isolated nucleoli, 194, 195
 protein content
 of isolated nuclei, 194, 195
 of isolated nucleoli, 194, 195, 217, 218
 RNA content
 of isolated nuclei, 194, 195
 of isolated nucleoli, 194, 195
- Spermatocytes
 cytidine incorporation in locust, 348, 349
 Drosophila, 150, 152
 Gryllidae
 association nucleolus of, 6, 77
 sex chromosome of, 6, 77
 RNA formation in *Drosophila*, 152
- Spider oocyte nucleolus
 DNA content of, 246, 247
 protein content of, 246, 247
 RNA content of, 246, 247
- Spinal cord, nucleolonema of, 103
- sRNA, *see* Transfer RNA
- Starch gel electrophoresis, of nucleolar proteins, 210
- Starfish, *see* *Asterias*
- Sulfhydryl groups, concentration in nucleolus, 205, 206
- Surface area, relationship to nucleolar volume, 423
- Synchronous cell cultures
 of CMP cells, 355, 356
 ribosome synthesis in, 560, 561
 rRNA synthesis in, 560, 561
- Syringomyelia, 95
- T**adpole, *Xenopus*-
 DNA content of, 299
 RNA content of, 299
- Tailbud stage, nucleolar size in, 301
- Tegenaria*
 cytoplasmic nucleotide composition of, 226
 DNA nucleotide composition of, 226
 nucleolar nucleotide composition of, 226
- Telophase
 nucleolus
 in *Allium cepa*, 129, 130
 formation of, 69
 metachromatic staining of, 129
 RNA on chromosomes, 152
 in root meristems, 350
- Terminal incorporation
 of adenine in nucleolar RNA, 226
 of cytidine in nucleolar RNA, 226
- Testis, DNA nucleotide composition in *Chironomus*, 226
- Thioacetamide effects
 on nuclear enzymes, 376
 on nucleolus, 369
 morphology, 23
 ribonuclease, 289, 290, 293
 RNA content of, 372, 373
 synthesis in, 372, 373
 volume of, 372, 373
 on RNA
 35S, 199, 200
 45S, 199, 200
 55S, 199, 200
 high molecular weight, 378
- Thymidine incorporation
 in ascites cells, 36, 37
 in cell cycle, 339, 340, 346, 347
 in HeLa cells, 498, 500
 into intranucleolar chromatin, 19
 into monkey kidney nucleoli, 19
 into nucleoli, 36, 37, 394, 400, 401
 into nucleolus-associated chromatin, 19
- Thymocytes, nuclear protein synthesis in, 224
- Tissue culture cells
 exhibiting anisonucleoliosis, 168
 exhibiting isonucleoliosis, 168
 nucleohistones of nucleolus-associated chromatin, 16
- Toluidine blue molybdate (TBM), RNP stain, 167, 168
- Transfer RNA, *see* RNA, Transfer
- Translation, sea urchin embryo
 first period, mRNA response, 325, 326
 second period, mesenchyme blastula, 325, 326
- Transmethylation, RNA, 257, 263, 264
- Transplantation
 between NO⁻ and NO⁺ animals, 303
 of nuclei
 rRNA synthesis inhibition, 303
 in *Xenopus*, 303
- TRB
 effect of RNA polymerase transcription, 258, 259
 RNA synthesis inhibition of, 258, 259
 uridine incorporation inhibition of, 258

- Tricyamp, effect on nucleolar synthesis, 267
- Triton X-100, effect on ribonuclease, 289, 290
- Triturus* oocyte
 carrier RNA, 476
 cytoplasmic nucleotide composition of, 57
 DNA nucleotide composition of, 226, 230
- intranucleolar
 DNA necklace, 55, 57
 "membrane," 56, 58
- nuclear envelop morphology of, 54, 55, 57, 58
- nucleolus
 appearance in, 302
 fibers of cleaving eggs, 151
 fibrous core
 protein, 55
 RNA, 55
 fusion of, 414
 granular cortex
 protein, 55
 RNA, 55
 lampbrush chromosome stage morphology of, 54, 55, 56
 nucleotide composition of, 226
 RNA
 content of, 475, 483
 synthesis in, 54
- Tropocolum majus*, 72, 73
- Trypsin
 effect on nucleolar morphology, 56
 effect on ribosome activity, 312
- Tryptophane, incorporation in cell cycle, 355, 358, 359
- U**nfolding of nucleoli, 110
- Urechis*, 350
 accessory oocyte nucleoli of, 341, 342
 nucleolar volume of, 341
 prochromosomes of, 338
 RNA synthesis in oocyte of, 341
 spermatocytes of, 343
- Uridine incorporation
 autoradiography of
 Smittia salivary gland, 258
 Xenopus, 426, 427
 in Balbiani rings, 395
 in cell cycle, 190
 in Chironomid nucleolus, 395, 400, 401
 DRB inhibition of, 258
- effect of actinomycin in sea urchin oocytes, 314, 320
 in euchromatin, 19
 in HeLa cells, 493
 into heterochromatin, 19
 into intranucleolar chromatin, 19
 in monkey kidney cells, 19
 in nucleolonema, 19
 into nucleolus-associated chromatin, 19
 in nucleolus organizer mutant, *Xenopus*, 426, 427
 into nucleoplasm, 19
 recovery as ribothymidine, 264
 into 28S RNA, 259
 into rRNA, 259
 into tRNA, 259
 into 40S RNP, 437, 539
 into root tips, 182
 into salamander oocyte RNA, 478, 479, 480, 481
 into sea urchin oocyte, 312, 313, 315, 320
 in *Smittia* salivary gland nucleolus, 258, 259
 in tissue culture cells, 355
- Urodeles, tRNA synthesis in, 323
- Uterine protein synthesis, actinomycin effect on, 382
- UV irradiation, effect on nucleolar morphology, 22
- V**acuoles
 in *Allium cepa*, 128, 129
 fibrillar components in *Allium cepa*, 128, 129
 nucleolar, 52, 571
 in pars amorpha of *Allium cepa*, 132
- Vicia faba*, 413, 414
 root tip nucleoli of, 43
 tRNA in, 569
- Vinca rosea* alkaloids, effect on nucleolar morphology, 22
- Virus infection
 effect on nucleolini, 21
 hypertrophy of nucleoli, 21
 parainfluenza, 367
 RNA synthesis in nucleoli, 21
- Vitellogenesis, 398
- Volume of nucleolus
 effect of partial hepatectomy on, 370, 371
 in maize, 147
 in *Urechis* oocyte, 341
 in wheat, 147

- Walker tumor
nuclei
 amino acid content of histones of, 195, 204, 205
 DNA
 content of, 194, 195, 208
 nucleotide composition of, 195
 protein content of, 194, 195
 RNA
 aqueous phase nucleotide composition of, 196, 197
 content of, 194, 195
 interphase nucleotide composition of, 196, 197
nucleoli
 actinomycin autoradiograph of, 210
 amino acid content of histones of, 195, 204, 205
 DNA
 content of, 195, 246, 247
 nucleotide composition of, 195
 isolation of, 194
 protein content of, 194, 195, 246, 247
 RNA
 aqueous phase nucleotide composition of, 196, 197
 content of, 194, 195, 246, 247
 interphase nucleotide composition of, 196, 197
 45S turnover in, 204, 205
WI-38 diploid cell, nucleolini of, 170, 171
- Xenopus laevis*, see also Nucleolus Organizer
 Mutant of, 147, 151, 152, 184, 226, 301, 302, 350, 398, 422, 564
 embryo, nucleic acid content of, 425, 426
 methionine incorporation in, 427, 428
 nucleolar appearance in, 302
 oocyte RNA content of, 475, 476
 ribosomal DNA cistrons in, 227
 RNA
 18S, 152
 28S, 152, 257
 hybridization in, 300, 549
 tadpole, phenocopies of, 308
 uridine incorporation in, 426, 427
X-ray
 effect on nucleolar morphology, 22
 effect on offspring nucleolus organizer, 397, 407
- Yeast
 ribosomal protein synthesis in, 552
 ribosome synthesis in, 552
 RNA synthesis in, 552, 561
 Yolk platelets, contamination of isolated nucleoli, 243
- Zea mays*, see also Maize
 cytidine incorporation in, 348, 349





Amazing Research.
Amazing Help.

<http://nihlibrary.nih.gov>

10 Center Drive
Bethesda, MD 20892-1150
301-496-1080



3 1496 00026 3650

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Public Health Service